2 Ectomycorrhizal fungus inoculations: a tool for improving forestation practices

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Introduction

The need of many species of forest trees for ectomycorrhizal associations was initially observed when attempts to establish plantations of exotic pines routinely failed until the essential fungi were introduced (Briscoe 1959; Clements 1941; Gibson 1963; Hatch 1936; Kessell 1927; Madu 1967; van Suchtelen 1962). The need of pine and oak seedlings for ectomycorrhizae has also been convincingly demonstrated in the afforestation of former treeless areas, such as the grasslands of Russia and the Great Plains of the United States (Goss 1960; Hatch 1937; McComb 1938; Rosendahl and Wilde 1942; Shemakhanova 1962; White 1941).

The primary purpose for inoculating with symbiotic fungi in world forestry is to provide seedlings with adequate ectomycorrhizae for planting in man-made forests. Such treatment has proven essential in forestation of cutover lands and other treeless areas, introduction of exotic tree species, and reclamation of adverse sites such as mining spoils. Use of ectomycorrhizal fungi can be of major significance to artificial regeneration.

Most research on inoculation with ectomycorrhizal fungi has been based on two working premises. First, any ectomycorrhizae on roots of tree seedlings is far better than no ectomycorrhizae at all. Success in correcting these deficiencies has contributed greatly to our understanding of the importance of ectomycorrhizae to trees, especially as they relate to the establishment of exotic forests. Secondly, some species of ectomycorrhizal fungi under certain environmental conditions are more beneficial to trees than other fungal species. Much more research should be aimed at selecting, propagating, manipulating, and managing the more desirable fungal symbionts to improve tree survival and growth.

The majority of past work on inoculation with ectomycorrhizal fungi has been done in nurseries for the production of bare-root or containerized tree seedlings. Most future work with inoculations will undoubtedly continue to concentrate on seedling production. However, the inoculation of seed for direct seeding operations could become a very important alternative to planting seedlings, especially on the more remote sites or those of very rough terrain.

Trappe (1977), Mikola (1973), and others (Bowen 1965; Imshenetskii 1955; Shemakhanova 1962) have thoroughly reviewed past work on ectomycorrhizal inoculations. I, therefore, am free to discuss selected reports as they relate to specific procedures and concentrate on recent published and unpublished work on pure culture manipulation of ectomycorrhizal fungi.

Ectomycorrhizae are formed by fungi belonging to the higher Basidiomycetes (mushroom and puffball group), Ascomycetes, and zygosporic Phycomycetes of the Endogonaceae (Gerdemann and Trappe 1974; Trappe 1962, 1971). The host plants of these fungi are predominantly trees belonging to the Pinaceae, Fagaceae, Betulaceae, Salicaceae, Juglandaceae, and other families (Meyer 1973).

Many species of fungi may be involved in the ectomycorrhizal associations of a forest, a single tree species, an individual tree seedling, or even a small segment of lateral root. As many as three species of fungi have been isolated from an individual ectomycorrhiza (Zak and Marx 1964). Even as a single tree species can have numerous species of fungi capable of forming ectomycorrhizae on its roots, a single fungus species can also enter into mycorrhizal association with numerous tree species. Some fungi are apparently host specific; others have broad host ranges and form mycorrhizae with members of numerous tree genera in diverse families (Trappe 1962).

The majority of reports on inoculations with ectomycorrhizal fungi involve Basidiomycetes on pines, oaks, and eucalypts. Several types of natural and laboratory produced inocula and methods of application have been used through the years. Many of these procedures have proved successful, others have not. Frequently, conflicting results are encountered.

Natural dissemination of spores

No ectomycorrhizal fungus in a natural environment has been shown to complete its life cycle in the absence of mycorrhizal association (Hacskaylo 1971). There also is no evidence to suggest that these fungi grow saprophytically in a natural forest soil (Shemakhanova 1962). However, once root infection has taken place extramatrical growth of mycelium from roots into large soil volumes is not unusual. Sporophores may occur dozens of metres from the above-ground parts of the tree host.

Most ectomycorrhizal fungi produce sporophores containing numerous spores. These spores can be disseminated great distances by wind, rain, insects, small animals, etc. The greater the density and closeness of the ectomycorrhizal tree hosts to the seedling producing areas, the greater the chances are for rapid natural ectomycorrhizal

development on the seedlings. In the southern United States, ectomycorrhizae appear in the spring on nursery-grown pine seedlings as early as six to eight weeks after seed germination. This occurs even in nursery soil fumigated just a few days prior to seeding because these nurseries are usually surrounded by dense stands of pine and oak which support abundant sporophores of mycorrhizal fungi. However, dry or cold weather often influences the fruiting habits of these fungi causing erratic spore production and dissemination. As mentioned by Trappe (1977), although heavy rains during the fruiting season stimulate fruiting, these rains can also restrict dispersal by washing spores from the air near their source.

The cultural procedures used to produce seedlings in bare-root or container nurseries create environmental conditions which select certain mycorrhizal fungi adapted to these conditions. In the southern United States, as well as other parts of the world, *Thelephora terrestris* appears to dominate the roots of most pines and oaks grown in nursery soil (Marx, Bryan, and Grand 1970; Mikola 1970; Weir 1921) and in containers (Marx and Barnett 1974).

In the southern United States, spore dissemination begins shortly after the nursery soil has been fumigated and seeded (usually from March to May) when *T. terrestris* produces sporophores and abundant spores in adjacent forests. These spores are carried by the wind to the fumigated soil, leach through the soil a few centimetres, and rapidly colonize the seedling roots.

This early colonization of seedling roots by *T. terrestris* can preclude colonization by other fungi which produce spores later in the year. These other fungi may form some mycorrhizae on the seedling roots later in the growing season, but only rarely do they dominate the roots. To maintain superiority on the roots, a fungus that appears first on seedlings must form mycorrhizae on short roots as rapidly as the short roots are produced. If it fails to do so other available fungi will infect these new roots. *Thelephora terrestris* and the other fungi which naturally occur and dominate seedling roots in nurseries have the capacity to spread rapidly under nursery conditions.

In nurseries in the United States and many other parts of the world, production of tree seedlings (particularly pine, oak, spruce, fir, and eucalyptus) is not seriously affected by deficiencies of ectomycorrhizae. In 1975, for example, there were over 1.2×10^9 coniferous tree seedlings produced in 190 nurseries in the United States. It is highly unlikely that these seedlings would have reached plantable size if they had not had adequate mycorrhizae. Ectomycorrhizal fungus deficiencies in properly managed tree nurseries in the United States, therefore, are rare.

The few mycorrhizal deficiencies reported in the United States in

recent times (Marx, Morris, and Mexal 1978; Trappe and Strand 1969) have appeared in newly established nurseries in which the soil was fumigated to control weeds and pathogens. Fumigation also eliminates residual inoculum of ectomycorrhizal fungi.

Not all deficiencies or the erratic occurrence of ectomycorrhizae on seedling roots are due to the absence of inoculum in soil. Excessive use of soluble fertilizers can reduce susceptibility of roots to infection (Bowen 1973; Marx, Hatch, and Mendicino 1977). In addition to fumigants, certain fungicides can significantly reduce or eradicate inoculum in soil and cause a deficiency of mycorrhizae on seedling roots (Hacskaylo and Palmer 1957; Iyer, Lipas, and Chesters 1971; Wojahn and Iyer 1976). However, it should be pointed out that certain fungicides can also stimulate development of ectomycorrhizae (Marx and Bryan 1969a; Powell, Hendrix, and Marx 1968), as can the application of certain nutrients to the soil which either stimulate residual inocula or host-root suscpetibility (Bowen 1973).

Natural inoculation of containerized seedlings grown in sterile or near-sterile potting mix is undoubtedly from airborne spores, but in many instances it is very erratic (Trappe 1977). In the southern United States pines are grown in a variety of containers for approximately three to four months (Balmer 1974). Natural ectomycorrhizal development on these seedlings is often erratic because they are watered and fertilized heavily to obtain the fastest possible growth in the shortest possible time. Unfortunately, these conditions induce high shoot–root ratios and low incidences of mycorrhizae on pine, both of which are thought to be undesirable for best field performance of seedlings (Marx and Barnett 1974; Marx, Hatch, and Mendicino 1977; Ruehle and Marx 1977). To ensure good ectomycorrhizae on containerized seedlings, procedures for inoculation with specific fungi and providing nutrients and water must be perfected (Ruehle and Marx 1977; Trappe 1977).

Cultural practices in nurseries influence the incidence of specific ectomycorrhizal fungi on tree seedlings. Levisohn (1965) reported that for many years *Suillus bovinus* was the only mycorrhizal fungus on seedlings of *Picea sitchensis* in certain nurseries in England. These nurseries were not fumigated and the soils were heavily composted each year with a variety of organic residues. The *S. bovinus* mycorrhizae soon disappeared from roots of *P. sitchensis* after outplanting unless the outplanting site was also heavily composted with organic matter. These results suggest that this fungus is not adapted to soils with low levels of organic matter. During subsequent years the nursery expanded and began to use different methods of fertilization in addition to amending the soil with organic residues. Associated with those cultural changes in the nurseries was the appearance of *Rhizo-*

pogon luteolus. Eventually this fungus dominated the spruce seedlings in the nursery, and thereafter *S. bovinus* occurred only rarely.

Soil or humus containing natural inoculum

The easiest and simplest method for eliminating ectomycorrhizal fungus deficiencies on seedlings in nurseries is to apply soil, humus, or duff containing mycorrhizae and associated mycelium. It is by far the most commonly used method to ensure consistent development of mycorrhizae (Mikola 1973). This form of inoculum can be collected from natural forests, plantations, or established tree nurseries. It is a very reliable method if done properly. The use of soil inoculum to propagate and maintain specific mycorrhizal fungi is unusual, but soil from truffle producing areas was used to inoculate seedlings in new areas (Malencon 1938). The success of this effort is unknown. In the Soviet Union (Shemakhanova 1962), soil containing mycorrhizae is routinely placed in holes prior to planting acorns for regeneration of oaks in shelterbelts. These areas of the Soviet Union are not devoid of ectomycorrhizal fungi, but apparently soil inoculation increases survival and early growth of the oak seedlings. The purpose of inoculation is to introduce new fungi into the area and enhance development of mycorrhizae on seedlings. The latter point is considered critical for artificial regeneration of oaks in Russia.

Mikola (1970) discusses in detail the various practices used in tropical and subtropical countries to ensure mycorrhizal development on nursery seedlings raised in various containers (pots, wooden boxes, plastic tubes, Swaziland beds, etc.). In most instances, 10 to 20 per cent of the container mixture is topsoil or humus containing mycorrhizae collected from a healthy pine plantation or established nursery. In order to conserve soil inoculum a small quantity of soil is sometimes added to the base of individual seedlings in containers.

A major drawback to the use of soil or humus as inoculum is that the specific fungi in the mixture cannot be controlled. Also there is no assurance that the chosen soil inoculum contains the most desirable fungi for the tree species being produced. The large volumes of soil needed to inoculate a nursery creates a logistics problem since 10 per cent of the volume of soil is currently recommended to assure adequate inoculation of nurseries (Mikola 1973). This volume of mycorrhizal soil can be extremely large in a nursery covering many hectares. Soil inoculum may contain a variety of harmful micro-organisms and noxious weeds in addition to the ectomycorrhizal fungi. Some of these micro-organisms may not be potentially harmful only to the tree seedling crop (Mikola 1973) but possibly to nearby agricultural crops (Marx 1975).

Ectomycorrhizal seedlings or excised ectomycorrhizae

Tree seedlings with ectomycorrhizae or excised mycorrhizae have been used as inoculum for new seedling crops. Chevalier and Grente (1973) were able to successfully establish the truffle fungus *Tuber melanosporum* in nursery beds from seedlings previously inoculated with this fungus. New seedlings growing adjacent to the pre-inoculated seedlings formed *T. melanosporum* ectomycorrhizae. Mikola (1973) discussed the Indonesian technique of inoculation. Seedlings with abundant mycorrhizae are planted at one to two metre intervals in new seedbeds. This technique is highly successful in forming ectomycorrhizae on seedlings of *Pinus merkusii.* Levisohn (1956) used surface-sterilized pine roots with *Rhizopogon luteolus* mycorrhizae to successfully form mycorrhizae on and stimulate growth of *Pinus contorta* seedlings in pots. Ekwebelam (1973) inoculated *Pinus caribaea* var. *hondurensis* and *P. kesiya* by growing them for three months in polyethylene bags filled with sterile sand containing excised mycorrhizae formed by *Rhizopogon luteolus.* The typical white coralloid ectomycorrhizae usually associated with *R. luteolus* was observed on roots of the seedlings within one month of germination. Ekwebelam did not mention non-inoculated control seedlings in his experiment, but in his previous experiments non-inoculated seedlings grown under similar conditions in this area of Africa apparently remained free of ectomycorrhizae.

Procedures using seedlings with ectomycorrhizae or excised mycorrhizae may be useful in propagating a specific fungus if soil conditions maintained in the nursery favour the introduced fungi and not those that occur naturally. Unless the introduced fungus is more adapted to nursery conditions than the naturally occurring ones, the desired fungus will eventually be displaced from roots.

Sporophores and spores

According to Trappe (1977), the first attempts to use specific fungi to form mycorrhizae on seedlings dates back to the eighteenth century. Sporophores of truffle fungi were added to planting holes of oak seedlings in new plantations in attempts to enhance truffle production (Malencon 1938). These inoculations took place nearly 75 years before the term 'mycorrhiza' was coined and over 100 years before the true nature of ectomycorrhizal associations was demonstrated. Unfortunately there is no way of determining to what degree these inoculations were successful. Sporophores of various ectomycorrhizal fungi, such as *Pisolithus tinctorius* and *Rhizopogon luteolus*, have been dried and/or chopped into small pieces and used

to infest soil successfully (Donald 1975; Fontana and Bonfante 1971; Mullette 1976). Fresh sporophores have also been added to soil inoculum prior to its use in nurseries to enhance the infective capacity of the soil (Mikola 1973). Inoculum composed of whole or chopped sporophores is basically nothing more than spore inoculum, since the vegetative matrix of the sporophores undoubtedly decomposes shortly after incorporation into soil.

Portugal

In 1970, Azvedo (personal communication) began developing a technique of seed inoculation using dried sporophores of different ectomycorrhizal fungi. Sporophores of *Amanita muscaria, A. phalloides, Boletus granulatus, B. scaber, Hydnellum zonatum, Lactarius delicious, L. chryzoreus, Lepiota procera, Russula cyanoxantha, Sarcodon imbricatum,* and *Tricholoma terreum* were collected fresh and dried carefully in the laboratory for one week. They were then transferred to a dessicator maintained at 30 °C for a few more days to complete the dehydration, crushed into a fine powder, and stored in sealed polyethylene bags. Seeds of *Pinus pinaster* were moistened with water and coated with the dried inoculum. After six to eight months in greenhouse tests using steamed soil in pots, *A. muscaria, R. cyanoxantha, S. granulatus,* and *H. zonatum* were found to be the most efficient fungi in forming typical ecto- and ectendomycorrhizae on *P. pinaster.* In another test, *R. cyanoxantha, T. terreum,* and *B. granulatus* formed the most ecto- or ectendomycorrhizae on *P. pinaster* after three months in Japanese paper pots. Control seedlings from non-inoculated seed, in most instances, remained free of any type of mycorrhiza. Azevedo states that this dried form of inoculum remains viable for four to five years when properly stored. Again, we can assume that the functional portion of the dried sporophores is basidiospores and not mycelium. These dried basidiospores of various fungi survived considerably longer than basidiospores of *Rhizopogon luteolus* in other experiments.

Australia

Pryor (1956) added basidiospores of *Scleroderma flavidum* to heat-sterilized soil in pots. Abundant ectomycorrhizae formed on roots and growth of *Eucalyptus dives, E. pauciflora,* and *E. macrorrhyncha* was stimulated. From these results he concluded that the absence of ectomycorrhizae on these *Eucalyptus* spp accounted for regeneration failures in Iraq and other parts of the world.

Theodorou (1971) found that inoculation of seeds of *Pinus radiata* with freshly harvested basidiospores of *R. luteolus* was an easy and effective way of introducing mycorrhizal fungi into both sterile and

non-sterile soil (mycorrhizal fungus deficient) in pots and in the field. This technique involved soaking surface-sterilized seeds of *P. radiata* in a sterile water suspension of basidiospores which coated each seed with approximately 1.9×10^6 spores. Theodorou found that more mycorrhizae formed on seedlings grown in sterilized soil than on those grown in non-sterile soil. He concluded that sterilization of soil enhanced mycorrhizal development by eliminating soil organisms deleterious to *R. luteolus*. Theodorou and Bowen (1973) later found that spores from freeze-dried sporophores of *R. luteolus* could be used to inoculate seed. Seed coated with basidiospores could be dried and stored (2 °C) for one month. They found that spore numbers must be increased by up to 100 times with freeze-dried spores and up to ten times with spores air-dried for two days to obtain ectomycorrhizal development on *P. radiata* seedlings equal to that of freshly collected basidiospores. Apparently freeze- and air-drying kills or inhibits germination of a substantial number of these basidiospores.

In pot studies Lamb and Richards (1974a,b) found that chlamydospores of three unidentified fungi were generally not as effective as basidiospores of *Rhizopogon luteolus, Suillus granulatus,* or *Pisolithus tinctorius* in forming ectomycorrhizae on *Pinus radiata* in natural soils lacking ectomycorrhizal fungi. The effectiveness of the different types of spore inocula, however, was improved by increasing inoculum density of the fungi or by increasing the amount of available phosphorus in the soils to 40 kg/ha. This stimulating effect of phosphorus is somewhat surprising since Mullette (1976) reported that basidiospores, i.e. crushed sporophores of *P. tinctorius*, would not form mycorrhizae on *Eucalyptus gummifera* in sterile quartz containing more than 3 kg of available P/ha (5 p.p.m.).

South Africa

Donald (1975) added air-dried and ground sporophores of *Rhizopogon luteolus* to fumigated soil in South Africa prior to seeding *Pinus radiata*. After eight months, seedlings from inoculated soil in one nursery had abundant white ectomycorrhizae with loose mycelium radiating from them. Sporophores of *R. luteolus* occurred in the inoculated beds and were associated with the white mycorrhizae. Donald concluded that the functional component of the dried sporophores was basidiospores and that they (4.4×10^7 spores per m^2 of soil surface) can be used as inoculum to form mycorrhizae on *P. radiata* in a conventional tree nursery.

United States

In recent years basidiospores of *Pisolithus tinctorius* have been used

in a variety of nursery and container tests on pines in the southern United States. Marx and Bryan (1975) added freshly collected basidiospores of *P. tinctorius* to fumigated soil in nursery microplots at a rate of $1.3 \times 10^{10}/m^2$ around two-month-old seedlings of *Pinus taeda*. *Pisolithus* formed approximately half of all the ectomycorrhizae on seedlings by the end of the growing season. At the time of soil infestation these seedlings had a few ectomycorrhizae formed by naturally occurring *Thelephora terrestris*. The identity of the different mycorrhizae is discussed later. This competition with *T. terrestris* for feeder roots may have accounted for the lack of dominance of *P. tinctorius* on the seedling roots. Competition between these fungi was observed recently on container-grown seedlings of *P. taeda*. Seedlings were inoculated at two, four, six, and eight weeks after germination with basidiospores of *P. tinctorius* (Ruehle 1980). The older seedlings which already had a few *T. terrestris* mycorrhizae at inoculation formed fewer mycorrhizae with *P. tinctorius* in the same period of time than younger seedlings inoculated with *P. tinctorius* before *T. terrestris* could colonize a substantial part of their root systems.

In these and subsequent experiments carried out by Institute scientists, the degree of ectomycorrhizal development is expressed as a percentage of all the short roots infected. Normally the introduced fungus occurs in mixtures on the roots with naturally occurring fungi. Therefore, the amount of mycorrhizae formed by the introduced fungi is expressed as a part of the total percentage of mycorrhizae formed.

Basidiospores of *P. tinctorius* have also been used in conventional nurseries in the southern United States. Following effective soil fumigation in two different tree nurseries, ectomycorrhizae were formed with basidiospores on seedlings of *Pinus taeda, P. elliottii* var. *elliottii, P. virginiana, P. clausa,* and *P. strobus* after one growing season. The freshly collected spores were incorporated into the soil at a rate of $2.55 \times 10^9/m^2$ of soil surface just prior to seeding. The success of soil infestation with the basidiospores varied. On *P. clausa* in Florida, *Pisolithus* accounted for about 12 per cent of all the ectomycorrhizae, and on *P. taeda* and *P. strobus* in North Carolina it accounted for nearly 70 per cent of all the ectomycorrhizae. Naturally occurring fungi formed the remaining mycorrhizae. *Pisolithus tinctorius* failed to dominate the root systems. More basic studies on *P. tinctorius* basidiospores (Marx 1976) revealed that even in a soil environment free of competing ectomycorrhizal fungi, it takes basidiospores at least two months after seed germination to form macroscopically detectable ectomycorrhizae and four months to stimulate growth of *P. taeda* seedlings. During this period other fungi obviously can

colonize roots in a natural soil. These studies also revealed that basidiospores collected from dry, insect-free sporophores can be stored in amber bottles at 5 °C for 34 months without loss of capacity to synthesize mycorrhizae (Marx 1976). Currently these spores are used for ectomycorrhizal synthesis; they have been stored for over five years under these conditions.

In the spring of 1975 basidiospores of *P. tinctorius*, as well as mycelial inocula of *P. tinctorius* and other fungi, were successfully used to correct the erratic occurrence of ectomycorrhizal fungi in a new tree nursery in south-eastern Oklahoma (Marx *et al.* 1978). Basidiospores were added to fumigated and non-fumigated soil just prior to seeding at rates of 1.19, 3.56, and 7.13×10^9 basidiospores per m^2 of soil surface. Seedlings of *P. taeda* formed abundant *Pisolithus* mycorrhizae in all plots after one growing season. There were, however, no well-defined differences in the amount of *Pisolithus* mycorrhizae formed in plots initially infested with different quantities of basidiospores. Basidiospores formed about 50 per cent more ectomycorrhizae on seedlings in fumigated soil than in non-fumigated soil. In fumigated soil, 70 per cent of all the mycorrhizae on seedlings were formed by *P. tinctorius*, whereas in non-fumigated soil *Pisolithus* accounted for less than half of all the mycorrhizae. Other ectomycorrhizae were formed by naturally occurring fungi. Fumigation eradicated these latter fungi and other microbial competitors, increasing the effectiveness of the *Pisolithus* basidiospores.

Another study was installed in the same nursery in the spring of 1976 to examine different practical methods of infesting soil with basidiospores of *P. tinctorius* (Marx, Mexal, and Morris 1979). Basidiospores (stored at 5 °C for eight months) were added to fumigated soil prior to seeding by (a) mixing spores in a hydromulch (wood pulp suspended in water) and broadcasting with a tractor-drawn applicator, (b) dusting spores onto the soil surface, or (c) injecting spores into soil with a tractor-mounted injector. Two other treatments were (d) dusting spores or (e) drenching spores onto seedlings six weeks after seeding. The rate of basidiospore application in all treatments was 5.5×10^8 per m^2 of soil surface. After one growing season the 350 000 seedlings of *P. taeda* were lifted and evaluated. Spores mixed with the hydromulch (a) were the most effective treatment. Three-quarters of the seedlings in this treatment had *Pisolithus* mycorrhizae and these represented over one-quarter of the total formed on the seedlings. This development resulted in a 15 per cent increase in the number of plantable seedlings and stimulated overall seedling growth (fresh weight) by 25 per cent over non-inoculated controls. The next best treatment was (b), dusting spores onto the soil at time of seeding. Only one-third of the seedlings in

this treatment had *Pisolithus* mycorrhizae, and these only represented about one-tenth of all the mycorrhizae on the seedlings. There were 13 per cent more plantable seedlings in this treatment and seedling fresh weights were approximately 12 per cent greater than the controls. There are problems in using dry basidiospores. During dusting the dry spores are difficult to control because breezes carry them great distances from the intended plot. This inconsistency of soil inoculation caused erratic development of mycorrhizae. All other methods of spore inoculation were not very effective. *Thelephora terrestris* formed abundant ectomycorrhizae on all seedlings in this study. Basidiospores of this fungus came from the numerous sporophores produced under pines planted adjacent to the nursery a few years earlier to provide a natural inoculum source (Marx *et al.* 1978).

Container-grown pine seedlings have been inoculated with basidiospores of *P. tinctorius* (Marx and Barnett 1974; Ruehle and Marx 1977). Root substrates such as vermiculite, peat moss, and pine bark, used in containers in the United States are successfully inoculated with mycorrhizal fungi because these substrates normally contain few microbial competitors. In greenhouse studies, equal success is achieved in forming *Pisolithus* mycorrhizae by dusting basidiospores onto seedlings (in a wind-free area) or mixing spores directly into the root substrate prior to seeding. Another promising technique is to mix basidiospores of *P. tinctorius* in the external matrix of encapsulated pine seed. For the past year the forest division of Hilleshög Seed Company Ltd., Landskrona, Sweden, and our research group in Athens, Georgia have been working co-operatively on the development of this technique. Encapsulation permits many spores to be placed on individual seed. However, the encapsulating material must be non-toxic to the spores and to the seed, and it must degrade rapidly after planting to permit satisfactory spore release onto the root zone.

It is obvious that spores of ectomycorrhizal fungi can be used in a variety of ways to either infest soil or inoculate seed for mycorrhizal development on seedlings in nurseries and containers. Results are not always positive, however. During the past eight years (Marx, unpublished data) basidiospores of a variety of fungi have been carefully collected, stored briefly, and used to infest steamed or fumigated soil in a special mycorrhizal fungus-free growth room in Athens (Marx 1973). With the exception of *P. tinctorius* and *T. terrestris*, basidiospores of *Amanita muscaria, A. caesarea, A. rubescens, Paxillus involutus, Lactarius deliciosus, L. piperatus, L. indigo, Laccaria laccata, Suillus luteus, Clitocybe nuda,* and *Russula emetica* did not form mycorrhizae on *Pinus taeda* or *P. echinata* seedlings in a four- to six-month test period. Trappe (1977) has also encountered difficulties

in forming mycorrhizae on western conifers with basidiospore inoculum of various fungi collected in the Pacific Northwest of the United States, as has Shemakhanova (1962) in Russia with various tests on oak. Obviously, a great deal more research is needed on collecting, storing, handling, and inoculating procedures for spores of ectomycorrhizal fungi before they can be successfully used in inoculation programmes.

Advantages and disadvantages

There are advantages and, unfortunately, certain disadvantages in using spores of ectomycorrhizal fungi for inoculation purposes. The major advantage is that they require no extended growth phase under aseptic conditions in the laboratory as does the production of vegetative mycelial inoculum (see later discussion). Another advantage is their lack of bulk. According to Donald (1975), there are approximately 11 million spores per gram of ground sporophores of *Rhizopogon luteolus*. Marx and Bryan (1975) report approximately 1.1×10^9 spores of *P. tinctorius* per gram of basidiospores. Large numbers of basidiospores can be collected from mature sporophores of many ectomycorrhizal Gasteromycetes such as *Pisolithus, Rhizopogon,* and *Scleroderma*. In less than 12 man hours, we have extracted 12 kilograms of basidiospores of *P. tinctorius* from sporophores collected from under young loblolly pines growing on a kaolin spoil in central Georgia. This one collection contained 12.5×10^{12} basidiospores. If these spores were used at a rate of 5.5×10^8 spores/m^2 of soil surface, this collection could be used to inoculate 5.5 million seedlings. It would be nearly impossible to collect this quantity of spores from any of the other ectomycorrhizal fungi, especially those belonging to the Agaricales or Aphyllophorales, but rapid collection is an advantage of *Pisolithus*. Another advantage of spores, at least those of certain fungi, is that they can be stored from one season to the next. This is important since spores collected in the summer or early autumn would normally have to be stored until the following spring if they are to be used to inoculate nursery seedlings.

There are also certain disadvantages in the use of spores as inoculum. Spores of many fungal species cannot be germinated to determine their viability. Hile and Hennen (1969) reported low germination of basidiospores of *P. tinctorius* on agar plates and were unable to make successful single spore transfers to new media. Lamb and Richards (1974c) tested different conditions of pH, temperature, and relative humidity and found that under the best test conditions only 0.38 per cent of the basidiospores of *P. tinctorius* would germinate. Basidiospores of other fungi, however, germinated much better than those of *P. tinctorius*. For years (Marx, unpublished data) various

physical and chemical stimuli were used to germinate basidiospores of *T. terrestris* and *P. tinctorius* without success. Apparently, synthesis of mycorrhizae is the only reliable means to determine viability of different spore collections of *P. tinctorius*. However, precise quantification of viable spores is difficult using the synthesis procedure (Marx 1976).

Other disadvantages are that the quantity of sporophores of many fungi required to inoculate nurseries may not be available every year and spore collections are frequently contaminated with various microorganisms. This is especially true of collections from Gastromycetes such as *P. tinctorius* where the basidiospores are exposed to the elements for several days or weeks during their maturation. Although data are not available, these contaminants may affect the health of tree seedlings or viability of spores.

The biggest disadvantage of using spores to inoculate seedlings is that it takes them several weeks to form mycorrhizae. This infection process is much slower than that achieved with mycelial inoculum (Marx, Bryan, and Cordell 1976; Theodorou and Bowen 1970). During this period of ingress less desirable fungi, such as pathogens (Marx 1972) or other ectomycorrhizal fungi, can colonize the roots and reduce the effectiveness of the introduced spore inocula. However, in parts of the world where the occurrence of ectomycorrhizal fungi is erratic or deficient, this delay may not have a significant effect on the final amount of mycorrhizae developed on tree seedlings from spore inoculum.

Mycelial inoculum

Ectomycorrhizal fungi as a group are difficult to grow in the laboratory. Many have never been isolated and grown in pure culture. Some species that have been isolated grow slowly, others often die after a few months in culture. Most ectomycorrhizal fungi require specific growth substances, such as thiamine, biotin, and simple carbohydrates, and are very sensitive to growth inhibiting substances (Palmer 1971).

The use of pure mycelial cultures of ectomycorrhizal fungi has been repeatedly recommended (Bowen 1965; Marx 1977a; Mikola 1973; Shemakhanova 1962; Trappe 1977) as the most biologically sound method of inoculation. Unfortunately, large scale nursery application of pure mycelial cultures has been severely hampered by the lack of sufficient amounts of inoculum. It may be possible to produce sufficient inoculum for research studies in small containers, pots, microplots, or even small nursery plots, but it is something else to produce a sufficient quantity of mycelial inoculum of an ectomycorrhizal fungus for a large nursery.

Another problem with pure mycelial cultures is knowing which fungal species to use under different conditions or with different hosts. During the past two decades a great deal of data on differences between mycorrhizal fungi and their differential effects on trees has been published (Bowen and Theodorou 1973; Marx 1977a; Theodorou and Bowen 1970). The first step in any nursery inoculation programme, therefore, must be the careful selection of suitable fungi (Bowen and Theodorou 1973; Mikola 1973; Trappe 1977).

Several researchers in various parts of the world have developed cultural procedures for producing pure mycelial inoculum of a variety of fungi for research purposes. In the last couple of decades, some of these procedures have been extensively used for various small experiments. Published information is available from Austria, Argentina, Australia, and, more recently, the United States. Experiments with pure culture inoculations have also been conducted in the Soviet Union, but details of these procedures or results have not been described (Levisohn 1958; Lobanow 1953; Mikola 1973). According to Wilde (1971), the use of pure cultures in the Socialist Republics in Europe have failed to produce significant results due to indigenous ectomycorrhizal fungi distributed throughout the soils. There are also numerous experiments with inconsistent or negative results. Since scientists tend to publish only positive results, experimental failures probably occur more frequently than we know from reviewing the literature (Mikola 1973).

Austria

Techniques used in Austria are based primarily on the work of Moser (1958a,b,c,d, 1959, 1961, 1963, 1965). Apparently techniques were developed initially to inoculate seedlings of *Pinus cembra* with low temperature strains of *Suillus plorans* in the nursery. This fungus was absent from the warmer nursery soils in the valley and in the alpine meadows, but it is a highly desirable fungal symbiont for the reforestation of this pine on the cold, high elevation sites near the timberline. Reforestation of these high elevation, mountainous areas is desirable in order to prevent avalanches and landslides.

For production of inoculum, *Suillus plorans* is first grown on Moser's (1958b) nutrient solution in small flasks for several days. The mycelium is transferred to 10-litre tanks containing the same nutrient solution and aerated for two to three hours daily for three to four months. The mycelium and liquid are poured into 5-litre flasks containing sterilized peat moss and fresh nutrient solutions. During the next few months, *S. plorans* grows throughout the substrate; the inoculum is then ready for use. Although attempts are made to maintain these cultures in aseptic condition, contaminations

by *Penicillium*, *Mucor*, and bacteria often occur. Moser (1963) refers to this contaminated inoculum as 'half-pure cultures' and claims that on certain occasions it proves more effective in forming mycorrhizae than pure cultures. He speculates that these contaminants add a 'rhizosphere effect' to the inoculum which is beneficial to ectomycorrhizal development and seedling growth. He also found that the most effective inoculum of *S. plorans* and other fungi is produced in organic materials such as sterile forest litter, ground peat, or sawdust. He observed best results with ground peat. Very inconsistent results were observed with agar inoculum or mycelial suspensions. Other workers (Ekwebelam 1973; Levisohn 1956; Mikola 1973; Marx, unpublished data) have used agar inoculum or mycelial suspensions with varying degrees of success.

Inoculum removed from the culture tanks is packaged in sterile polyethylene bags, transported to the nursery, and when possible applied to nursery soil within three days. The inoculum is usually placed in 10 cm deep furrows in the soil at a rate of 3 to 4 litres of inoculum per m^2 of soil surface. Young (one-month-old) seedlings of *P. cembra* are then transplanted into these furrows. The best mycorrhizal development occurs on seedlings growing in soil previously sterilized with heat or formalin.

Moser (1959) reported other means of using pure mycelial inoculum. It can be broadcast 1 cm deep onto soil and then chopped 10 cm deep into the soil prior to seeding. This method requires much larger amounts of inoculum (8–10 l/m^2 of soil surface) and the inoculum often dehydrates on the soil surface prior to its incorporation. With this method the inoculum must be able to survive in soil for the elapsed time between seeding and when short roots are formed on seedlings. With certain tree species in Austria, ectomycorrhizae may not form for eight months following soil infestation and seeding. This means that inoculum added to soil at sowing must be able to survive a rather long period in the absence of a host. Transplanting seedlings into furrows containing inoculum is preferred because it eliminates this problem. Since transplanted seedlings of certain tree species must remain in the nursery for two years (*P. cembra* is grown in the nursery for up to four years), there is the option of only inoculating every third or fourth row of seedlings. According to Moser (1963), once root infection has taken place the introduced fungi spread to adjacent seedlings. Another method of soil inoculation is placing inoculum in furrows between rows of established seedlings. This method is successful, but not recommended because digging furrows near seedlings can damage roots.

Moser (1963) has also used this technique to produce mycelial inoculum of *Suillus placidus, S. grevillei, S. aeruginascens, Paxillus*

involutus, *Amanita muscaria*, and *Lactarius porninsis*, either alone or in mixtures. Although Moser only presented limited quantitative data from different fungi/tree species tests in nurseries (1958a,b,c,d, 1959, 1961, 1963, 1965), the results show the biological significance of the inoculation. In one of Moser's nursery tests in a sandy alluvial soil, pure mycelial inoculum of *Phlegmacium glaucopus* formed abundant ectomycorrhizae on spruce. The inoculated seedlings had a healthy green colour and were considerably larger than non-inoculated seedlings, which had chlorotic foliage and roots completely free of ectomycorrhizae. Moser failed to mention the form of inoculum used, the method of inoculation, or the duration of the nursery test. In other tests (Moser 1963), larch seedlings were grown in both sterilized and non-sterilized soil of different types inoculated with *S. grevillei*, *S. aeruginascens*, *L. porninsis*, and a mixture of the three fungi. A fifth treatment was a control without inoculation. After two years the non-inoculated seedlings in the non-sterile soil from a spruce forest had an ectomycorrhizal frequency of 56, which was similar to that of seedlings from non-sterile soil inoculated with the fungi. The mycorrhizal frequency of the fungal treatments varied from 53 to 72. However, the non-inoculated seedlings in sterilized soil did not form ectomycorrhizae, while those in the fungal treatments had mycorrhizal frequency rates of 18 to 79. These data proved the importance of soil sterilization as a prerequisite to the effective use of mycelial cultures. Although no mention was made of the type of soil sterilization used, it obviously was successful in eliminating the indigenous symbiotic fungi. In a similar test using soil from a meadow, Moser (1963) found a much lower mycorrhizal frequency on inoculated seedlings from the same fungal treatments, especially on those growing in non-sterilized soil. The meadow soil not only had fewer indigenous symbiotic fungi, but also had a reduced potential for mycorrhizal development following inoculations with the fungi. Moser (1959, 1963) discussed other nursery experiments but did not provide data on seedling growth or mycorrhizal development.

Recently in Austria, Göbl (1975), a co-worker of Moser, discussed the selection and culture of specific ectomycorrhizal fungi for nursery inoculations and methods of producing sufficient amounts of inoculum for practical use. She generally follows the procedures of Moser and recommends growing the fungi in a liquid medium until adequate mycelium is obtained. This mycelium is placed in 1-litre bottles containing cooked and sterilized cereal grains such as wheat or white millet. Calcium sulphate (0.4 to 0.5 g/100 g of grain) is added to improve the growth of certain fungi. These grain cultures are shaken lightly each week and after two to four weeks at 20 to 22 °C

the grains become thoroughly colonized by the fungi. The grain cultures can then be stored at 4 to 6 °C for up to nine months. Göbl recommends that the grain culture be checked periodically for microbial purity on an appropriate agar medium.

The grain cultures are added to enriched peat moss for the final stage in the production of inoculum. The peat must be enriched with nitrogen and carbohydrates (ammonium tartrate, asparagine, soyabean meal, blood meal, malt extract, glucose), as well as inorganic nutrients in different combinations. The kinds and amounts of these supplements vary according to the species of fungus grown. Usually 7 to 10 grams of glucose per litre of peat is used as a standard for carbohydrates. Ten to 15 litres of sterile, enriched peat moss is placed in large transparent plastic bags and inoculated with a generous supply of grain culture. The plastic bags are plugged with cotton to provide aeration and are shaken occasionally during storage at 20 to 22 °C. After three to six weeks the inoculum is ready for use in the nursery. Contaminated cultures are apparently discarded. This method has been used to produce inoculum of *Suillus plorans, S. grevillei, Boletinus cavipes, Amanita muscaria,* and *Hebeloma crustuliniforme.* An interesting idea presented by Göbl (1975) was that the last phase involving the sterile peat moss could be done at the nursery. The problems created by shipping large volumes of peat moss inoculum would be eliminated by shipping just the grain cultures to the nursery.

After satisfactory inoculum has been produced it can be used to infest soil according to the various procedures of Moser (1963). Göbl (1975) recommended another unique method, which is to transplant a young tree seedling directly into inoculum contained in a larger volume of peat moss. After the peat moss supporting the seedling becomes colonized by the introduced fungus it is used for inoculum. Göbl (1975) prefers this form of inoculum to forest litter because it eliminates the introduction of unknown microbial populations into the nursery.

Argentina

Techniques used in Argentina were developed by Takacs (1961, 1964, 1967) at the Mycorrhiza Laboratory of the Instituto Nacional de Tecnologia Agropecuaria (INTA) at Castelar. When new pine nurseries are established in formerly treeless areas lacking ectomycorrhizal fungi the soil is inoculated with pure mycelial cultures. Techniques are very similar to those developed by Moser in Austria. Basidiospores or pieces of tissue from the sporophores are cultured on an appropriate agar medium. The mycelium is transferred to liquid culture, incubated, and added either to sterilized, germinated grains of cereals

(such as barley), the cereal chaff, a mixture of grain and chaff, or sterilized peat moss. All substrates are enriched with a liquid medium. Takacs (1967) inoculated the substrates either with mycelial agar discs or mycelium from liquid culture. This inoculum, regardless of the physical media, is used after one to two months' incubation at room temperature. Peat moss is used more commonly than the other substrates. Pure mycelial cultures of *Amanita verna, Suillus granulatus, S. luteus, Hebeloma crustuliniforme,* a *Russula* sp, *Scleroderma verrucosum,* and *S. vulgare* have been produced by this method and are apparently available from the INTA in Argentina (1967). The details for large scale nursery inoculation in Argentina using Takacs's method were described by Mikola (1969). Usually five 200-ml flasks of each of four different fungi contained in either peat moss or grain-chaff inoculum are sent from INTA to a nursery. Upon arrival at the nursery the contents of each of these 20 flasks are mixed with 4 to 10 kg of sterilized soil or forest litter. These mixtures are kept moist and incubated for three weeks before use in the nursery beds. Using this method, twenty 200 ml 'starter' cultures can be used to produce 100 to 200 kg of soil inoculum. According to Mikola (1969), this is sufficient to infest 500 m^2 of nursery soil. Inoculum is usually added to the soil during preparation of the nursery beds.

Since quantitative data are not available for this work it is difficult to evaluate the success of this method on a large nursery scale. However, based on our current knowledge it is difficult to believe that these fungi can grow saprophytically in the sterile soil or litter at the nursery. Mycelial growth must occur in the presence of competitive micro-organisms and in the absence of essential nutrients. If the starter inoculum survives the incubation in soil or litter at the nursery, perhaps all that is really accomplished is a dilution of the original inoculum. This diluted inoculum must be sufficient to effectively colonize seedling roots in these nurseries containing few, if any, ectomycorrhizal fungi which can compete with the introduced inoculum.

There is only limited quantitative data available to this author on the earlier experimental work in Argentina preceding the broad application of mycelial inoculations. In one test inoculation was done with mycelial discs from Petri dish culture and not with inoculum prepared by any of the previously mentioned methods. Takacs (1964) isolated *Scleroderma vulgare* from sporophores collected from a plantation of *Pinus taeda* and grew it on an agar medium. A nursery soil was mixed 4:1 with sand, sterilized with methyl bromide and 10 per cent formalin, and planted with seed of *P. taeda.* Sixty days after seed germination half the seedlings were inoculated with pieces of agar containing the fungus. These mycelial pieces were placed 10 cm apart and 3 to 4 cm deep in the soil. When all the seedlings were

lifted and measured after ten months, the results proved the value of inoculation. Inoculated seedlings formed abundant ectomycorrhizae and the soil from which they were lifted was a grey colour, apparently caused by the grey mycelium of *S. vulgare* colonizing the soil. Total fresh weights of the inoculated seedlings were 83 per cent greater than the non-inoculated controls. The differences were highly significant based on statistical analysis. Although it was not mentioned, it is assumed that seedlings in non-inoculated soil were free of mycorrhizae.

In an earlier test, Takacs (1961) used grain cultures of different fungi. Pure cultures of *Suillus granulatus, Scleroderma vulgare, Amanita phalloides,* and a *Russula* sp were obtained by germinating the spores on a liquid medium. The mycelium was used to inoculate sterilized, germinated barley seed. After only five days of incubation the grain cultures, according to Takacs, were ready to be used as inoculum. *Pinus pinaster, P. radiata,* and *P. thunbergii* were seeded in non-sterilized soil in a nursery bed. After 30 days, one or two grains of barley colonized by the specific fungi were placed 20 cm apart in the row next to the seedlings roots. Grain cultures of the four fungi were placed alternately in the rows. Apparently the seedlings were harvested several months later in the autumn. No quantitative data on mycorrhizal development was presented in this report but numerous ectomycorrhizae were illustrated. Takacs (1961) simply stated that the inoculated seedlings had exceptional growth and were considerably larger than the non-inoculated seedlings. No mention was made of other ectomycorrhizal fungi. It is difficult for this author to understand how these fungi spread rapidly enough from grain cultures placed so far apart to produce mycorrhiza on all seedlings. Another test was installed the following spring in different nurseries using the same techniques. Grain cultures of these four fungi were mixed together into non-sterile soil of three nurseries just prior to seeding. Germination began normally, but in two nurseries damping-off destroyed nearly 50 per cent of the seedlings in inoculated plots. The grain cultures probably contributed to the development of the damping-off micro-organisms. The seedlings were evaluated after six months. Takacs stated that the larger green seedlings had abundant ectomycorrhizae and the smaller chlorotic seedlings lacked mycorrhizae. However, he failed to report whether the large seedlings with ectomycorrhizae came from inoculated plots.

Australia

Theodorou (1967) developed pure mycelial inoculum of *Rhizopogon luteolus* using techniques similar to those of Moser. The purpose of inoculation with *R. luteolus* was to correct the deficiency

of ectomycorrhizal fungi in some Australian soils and also to produce seedlings from *Pinus radiata* with a root system having a greater capacity to absorb phosphorus from soil. Earlier work by Bowen (1962) showed that *P. radiata* seedlings had a better uptake of phosphorus with mycorrhizae formed by *R. luteolus* than with other fungi. Pure cultures were produced in a medium of vermiculite, chaff, and corn meal in a ratio of 10:2:1 moistened with a liquid medium. The fungus was placed in bottles containing about 80 grams of medium and incubated for one month at 25 °C. Twenty-five grams of inoculum were buried 8 cm deep in soil contained in pots which had either been steamed, fumigated with different rates of methyl bromide, or non-sterilized. Certain pots were reinoculated with 10 grams of non-sterile soil. All pots were seeded with *P. radiata* and seedlings were evaluated after nine months. Since Theodorou (1967) did not use non-inoculated, sterilized soil as a control, we must assume that all ectomycorrhizae were formed by the introduced fungi and not from naturally occurring fungi. In steamed or methyl bromide sterilized soil that was not reinoculated with non-sterile soil, mycorrhizal development varied from 33 to 41 per cent. Ectomycorrhizal development varied from 17 to 31 per cent in sterilized soil containing *R. luteolus* as well as the non-sterile soil. Substantial increases in growth of *P. radiata* seedlings were correlated with mycorrhizal development. Best growth occurred in sterilized soil containing only inoculum of *R. luteolus*. Theodorou concluded that the effectiveness of *R. luteolus* mycelial inoculum is suppressed by antagonistic soil organisms and, therefore, recommends sterilization of soil prior to artificial inoculation with this fungus. In another greenhouse study, Theodorou and Bowen grew freshly collected cultures of *Suillus granulatus, S. luteus, Cenococcum graniforme,* and *Rhizopogon luteolus* in vermiculite medium for three weeks as described above. The inoculum was mixed into the upper 8 cm of steamed soil contained in pots. A non-inoculated, sterile soil control was used. All pots were seeded with *P. radiata.* The study was terminated after 14 months and seedlings evaluated. Ectomycorrhizal assessments were done both macro- and microscopically. The degree of mycorrhizal development on seedlings inoculated with *R. luteolus, S. granulatus, S. luteus, C. graniforme,* and the controls was 20, 12, 16, 2, and 6 per cent, respectively. Dry weights of seedlings with *R. luteolus* were 90 per cent greater and those with *S. granulatus* were 30 per cent greater than the other three seedling groups. *Suillus luteus* and *C. graniforme* mycorrhizae did not stimulate seedling growth. Seedlings with *Rhizopogon* mycorrhizae contained 47 to 125 per cent more phosphorus in foliage than control seedlings or those with other fungi, showing the ability of mycorrhizae formed by this fungus to enhance phosphorus absorption in *P. radiata.*

In another test, Theodorou and Bowen (1970) produced inoculum of fresh cultures of *S. granulatus, S. luteus*, and four isolates of *R. luteolus* and inoculated soil sterilized by gamma irradiation. The pots were seeded to *P. radiata* and seedlings evaluated after two years. Although quantitative data on ectomycorrhizal development was not presented, the authors stated that all inoculated seedlings had very good ectomycorrhizal development and non-inoculated seedlings lacked mycorrhizae. Over 100 per cent differences in dry weights of seedlings were obtained between fungi. The growth of all inoculated seedlings was significantly better than the controls. There was as much as 85 per cent difference in dry weight of seedlings induced by different isolates of *R. luteolus*. As a group the *R. luteolus* isolates were superior to the other fungi in stimulating seedling growth.

United States

Tests to artificially introduce pure mycelial cultures of ectomycorrhizal fungi into soil were begun in the early 1930s by Hatch (1936, 1937). He grew seedlings of *Pinus strobus* in non-sterile prairie soil in large pots. These pots were housed in a chamber filtered to exclude contamination from air-borne spores of mycorrhizal fungi. Three months after seeding the seedlings were small, yellow, unthrifty in appearance, and devoid of mycorrhizae. Half of the seedlings were inoculated with agar cultures of *Suillus luteus, Boletinus pictus, Lactarius deliciosus, L. indigo,* and *Cenococcum graniforme.* After five months, root evaluations revealed that *S. luteus* and *L. deliciosus* formed mycorrhizae on 30 per cent of the short roots and stimulated seedling growth. The other fungi apparently failed to form mycorrhizae. Non-inoculated seedlings remained stunted and chlorotic. Hatch proved that pure cultures of specific fungi could be used to correct the deficiency of mycorrhizae. Once the natural soil lacking mycorrhizal fungi was inoculated and mycorrhizae were formed, it supported normal growth of white pine seedlings.

Three decades passed before Hacskaylo and Vozzo (1967) initiated a series of inoculation experiments in Puerto Rico with pure mycelial cultures of various fungi. In one test (Vozzo and Hacskaylo 1971) pure mycelial inocula of *Cenococcum graniforme, Corticium bicolor, Rhizopogon roseolus,* and *Suillus cothurnatus* were used. These fungi were selected because they were proven symbionts and had distinctive hyphal colours which should aid in subsequent evaluations. Following Moser's (1963) general technique, they grew the fungi on agar and then in liquid media. The mycelium from liquid culture was used to inoculate polypropylene cups containing a 2:1 ratio of sterile peat moss and vermiculite moistened with a glucose-ammonium tartrate nutrient solution (pH 3.8). After 16 weeks of incubation the inoculum

was flown from the USDA, Forest Service, Pioneering Research Unit Laboratory in Beltsville, Maryland, to Puerto Rico. In Puerto Rico, seedlings of *Pinus caribaea* were grown in a non-mycorrhizal condition for four months in a container nursery where they were watered and lightly fertilized. Seedlings were grown in 8 × 15 cm plastic bags filled with a 1:1 mixture of fumigated peat moss and vermiculite. The plastic bags were split and one-half cup of inoculum was placed against the exposed non-mycorrhizal roots of each seedling. The bag was closed and slipped into another container to hold the inoculum and root substrate intact. Ten months later the seedlings were measured and evaluated for mycorrhizal development. Certain seedlings were outplanted in the field; these results will be discussed later. Although they did not present quantitative data on mycorrhizal development, Vozzo and Hacskaylo (1971) reported that *Corticium bicolor, Rhizopogon roseolus,* and *Suillus cothurnatus* formed mycorrhizae. *Cenococcum graniforme* did not form mycorrhizae. Seedling height growth was correlated with ectomycorrhizal development, i.e. seedlings with the most ectomycorrhizae (*Corticium bicolor*) were the tallest. This study and others in Puerto Rico were complicated by the frequent occurrence of *Thelephora terrestris* sporophores and mycorrhizae throughout the nursery and on test seedlings. This fungus was introduced from the United States in pine duff inoculum in 1955 to correct the chronic deficiency of mycorrhizal fungi in Puerto Rico (Briscoe 1959).

Mycorrhizal Institute

In the south-eastern United States, formal research on the use of pure mycelial cultures began in 1966 at the USDA, Forest Service Laboratory in Athens, Georgia. In 1976, the research unit was given greater research latitude and was designated the Institute for Mycorrhizal Research and Development. One of several research goals of this multidisciplinary unit is to perfect existing techniques and to devise new ones for artificially inoculating tree seedlings with pure cultures of ectomycorrhizal fungi in bare-root and container nurseries. It is anticipated that these techniques will be valuable for not only correcting the erratic occurrence of ectomycorrhizal fungi in nurseries, but show the biological feasibility and practical value of manipulating and managing specific, highly desirable, ectomycorrhizal fungi on tree seedlings to improve the survival and growth of seedlings on routine and adverse reforestation sites.

Selecting fungi. Initial research efforts were concentrated on *Pisolithus tinctorius, Thelephora terrestris, Cenococcum graniforme*, and a few other fungal species. *Pisolithus tinctorius* was chosen because it

is readily propagated in the laboratory on a variety of agar or liquid media. It had yellow-gold hyphae and mycorrhizae which aid in its detection and quantitative assessment on seedling roots. The main reason for selecting this symbiont, however, was its apparent ecological adaptation to adverse soil conditions such as those found on coal spoils (Marx 1977a; Schramm 1966). *Pisolithus tinctorius* is also widespread on trees growing on kaolin spoils, sheet-eroded soils, borrow pits, and other biologically hostile sites. These sites are characterized by one or more adverse soil conditions—high soil temperatures, extreme acidity, high levels of Al, Mn, S, Fe, and chronic low fertility—which limit routine reforestation.

Seedlings with *Pisolithus* mycorrhizae formed in the nursery prior to outplanting on adverse sites should survive and grow better than routine nursery seedlings having *Thelephora* or other mycorrhizae. Tree seedlings 'tailored' with *P. tinctorius* should have a physiologically and ecologically adapted root system capable of surviving and persisting in adverse soils. The concept of forming mycorrhizae on seedlings with fungi ecologically adapted to the planting site parallels that proposed by Moser (1963) who used mycorrhizae formed by *Suillus plorans*, a low temperature fungus, on *Pinus cembra* to enhance reforestation of the high, cold, elevation sites in Austria.

There are results from some basic studies which help explain the persistence of *P. tinctorius* on these sites. In controlled temperature studies (Marx, Bryan, and Davey 1970) it was found that *P. tinctorius* was tolerant of high temperatures. The fungus grew in agar culture over a temperature range of 7 to 40 °C with an optimum at 28 °C. Later Momoh and Gbadegesin (1975), using a Georgia isolate of *P. tinctorius*, successfully grew mycelium of the fungus at 42 °C with an optimum at 30 °C. Lamb and Richards (1971) reported that the thermal death point for hyphae of *P. tinctorius* was 45 °C, whereas hyphae of *Rhizopogon luteolus* and other fungi were killed at 38 °C. Marx, Bryan, and Davey (1970) also reported that *P. tinctorius* formed more ectomycorrhizae on seedlings of *Pinus taeda* grown in aseptic culture at 34 °C than at lower temperatures. Later Marx and Bryan (1971) produced aseptic seedlings of *P. taeda* with different ectomycorrhizae at 25 °C and exposed them to higher temperatures. Seedlings with *Pisolithus* mycorrhiza had better survival and growth at 40 °C than non-mycorrhizal seedlings or seedlings with *Thelephora* mycorrhiza. *Pisolithus tinctorius* is also tolerant of and can be stimulated by the heavy metals often found in adverse sites such as coal spoils. Hile and Hennen (1969) found that the addition of iron and sulphur to agar medium stimulated vegetative growth of *P. tinctorius*. Muncie, Rothwell, and Kessell (1975) detected large amounts of elemental sulphur in the interior of sporophores of the fungus

collected from coal spoils. These authors suggested that *P. tinctorius* has a unique, but unexplained, method of utilizing sulphur. These various studies showing the high temperature and metal tolerance of *P. tinctorius* help explain its ability to survive on coal spoils and other adverse sites.

There are several other unique features of *P. tinctorius* which make it a potentially strong fungal candidate for practical use in a variety of different forest situations. In addition to its occurrence on adverse sites, it also occurs in urban areas, orchards, and routine forest sites. It has been reported to occur in tree nurseries (Marx 1977b), especially on two- to three-year-old pine seedlings in northern nurseries of the United States (Marx, unpublished data). *Pisolithus tinctorius* has a proven tree host-range of nearly 50 species and is associated with an additional 25 tree species. These trees include most of the world's more important species. This fungus occurs in 33 countries of the world and in 38 states in the United States (Grand 1976; Marx 1977b). Problems with plant quarantine regulations regarding the use of *P. tinctorius* in most parts of the world should be minimal because of its broad, natural geographic distribution. After considering all of these features it becomes apparent that the development of inoculum and inoculation techniques with this fungus might prove useful not only in reclamation of adverse sites, but in reforestation of routine forest sites with both indigenous and exotic tree species in various parts of the world.

Thelephora terrestris was selected for testing at the Mycorrhizal Institute because it also grows rapidly in the laboratory on a variety of agar and liquid media. Its hyphae and ectomycorrhizae on pine are white to cream-brown in colour (Marx and Bryan 1970; Marx, Bryan, and Grand 1970). Unfortunately this colour characteristic is common to a number of other ectomycorrhizal fungi and, therefore, visual detection and quantitative evaluation of *Thelephora* mycorrhizae are difficult. This fungus does, however, fruit on seedling stems or adjacent to seedlings on soil in nurseries and can be readily traced from sporophores to mycorrhizae via visible hyphal strands. This fungal symbiont is also widespread on tree seedlings in nurseries, as previously described, and has a broad host range (Hacskaylo 1965; Marx and Bryan 1969b, 1970; Weir 1921). Its common occurrence in nurseries suggests that it is ecologically adapted to the good tilth, fertility, and moisture conditions of nursery soils. Obviously *T. terrestris* is one of the major ectomycorrhizal fungi on roots of the enormous numbers of tree seedlings produced in nurseries and planted out annually on the millions of hectares of reforestation and reclamation sites all over the world. Its occurrence in nurseries also indicates that it is a major competitor to introduced inoculum of other fungi for seedling roots.

Cenococcum graniforme has been tested on a more limited scale at the Institute than the previously mentioned fungi. It was selected because of its easily identified, jet-black ectomycorrhizae, broad host-range (Trappe 1964), and apparent drought and high temperature tolerance (Mexal and Reid 1973; Meyer 1964; Saleh-Rastin 1976; Worley and Hacskaylo 1959). This last characteristic suggests that it may be valuable to seedlings planted on sites having seasonal drought conditions. Unfortunately vegetative growth of *C. graniforme* in pure culture is very slow. Using pure mycelial techniques, various researchers have also encountered difficulties forming mycorrhizae with this fungus on various tree species (Hatch 1936; Marx *et al.* 1978; Theodorou and Bowen 1970; Vozzo and Hacskaylo 1971).

Pure mycelial culture. Our first attempts at producing pure mycelial cultures of *P. tinctorius, T. terrestris,* and *C. graniforme* were not very successful. None of these fungi would form mycorrhizae on seedlings of *Pinus taeda* from wheat grain cultures (Marx, unpublished data). Grain cultures were added to steamed soil in a 1:15 ratio in a mycorrhiza fungus-free growth room (Marx and Bryan 1969b; Marx 1973) and seeded to *Pinus taeda*. After five months none of the inoculated and control seedlings had mycorrhizae. Microscopic examination revealed that the grain cultures were colonized by saprophytic fungi and bacteria as early as three weeks after soil inoculation. We observed a great deal of damping-off of pine seed as did Takacs (1961). The high nutritive value of the boiled wheat contributed to its rapid colonization by saprophytes, which probably killed the ectomycorrhizal fungi. Our results do not support the claim of Park (1971) that grain cultures of *C. graniforme* can be used to inoculate nursery soil; our results from this and several other studies conflict with this broad recommendation. Our results with grain cultures also conflict with those of Takacs as discussed by Mikola (1973). Our grain cultures of the various fungi were decomposed so rapidly by saprophytic organisms that we doubt if grain cultures could be used at a nursery as a starter culture to inoculate sterile soil for the production of a large volume of inoculum.

Vermiculite and peat moss moistened with a modification of Melin-Norkrans medium (Marx 1969) with glucose instead of sucrose was found to be an excellent substrate for the production of mycelial cultures of these fungi. In one of our first tests (Marx and Bryan 1970), inoculum of *P. tinctorius* and *T. terrestris* was grown aseptically for four months at 25 °C in 1 litre volumes of a 28:1 ratio of vermiculite and peat moss moistened with nutrient solution. The inoculum was mixed in a ratio of 1:8 with an autoclaved mixture of soil:peat moss:vermiculite in the growth room. Seeds of various pine

species were planted, and after four months root evaluation revealed that *T. terrestris* formed mycorrhizae with 22 tree hosts and *P. tinctorius* with 14 tree hosts. All non-inoculated seedlings were free of mycorrhiza. This technique was successfully used later in the growth room in a study with *P. tinctorius* on *Pinus clausa* (Ross and Marx 1972), and again with *P. tinctorius* and *C. graniforme* on *P. echinata* (Marx 1973). In these and other studies, mycelium of *P. tinctorius* and *T. terrestris* usually completely permeated the vermiculite and peat moss particles after less than three months' incubation at 25 °C. However, because of the slow growth rate of *C. graniforme*, it is necessary to incubate this fungus for six to eight months under the same growing conditions to completely permeate the substrate.

Leaching of mycelial inoculum. Our first tests outside the growth room using the vermiculite-peat moss inoculum in soil fumigated with methyl bromide gave variable results. After only a few weeks in the greenhouse the inoculum was extensively colonized by saprophytic fungi and bacteria in a fashion similar to that observed with the wheat grain cultures. This saprophytic colonization was markedly reduced by leaching the inoculum with water before adding it to soil. Leaching removed the non-assimilated nutrients and thus reduces the food base essential for saprophytic colonization by the micro-organisms. We compared several methods and types of liquids (physiological saline, sterile distilled water, and tap water) for leaching inoculum. The method which proved to be the best involved placing 4 litres of inoculum in a double layer of cheesecloth and irrigating this for several minutes under cool tap water. Excess water is removed by squeezing the inoculum in the cheesecloth by hand. This reduces the inoculum volume by one-third. In addition to non-assimilated nutrients, a great deal of pigment (a rich brown pigment in the case of *P. tinctorius*) and small vermiculite particles are washed from the inoculum during leaching. Other researchers (Moser 1963; Takacs 1967; Theodorou and Bowen 1970; Vozzo and Hacskaylo 1971) did not leach mycelial cultures prior to soil inoculation and certainly must have encountered competition problems with colonizing micro-organisms.

Leached vermiculite-peat moss inoculum of *P. tinctorius* was used successfully in 1972 to form mycorrhizae on pine at a microplot tree nursery (Marx and Bryan 1975). Inoculum was mixed at a ratio of 1:8 with fumigated soil contained in wooden framed microplots. Control soil was infested with autoclaved mycelial inoculum of *P. tinctorius* to standardize soil fertility and tilth. The soil was planted with seed of *P. taeda* in April and mulched. Periodic examinations of seedlings revealed that the mycelial inoculum of *P. tinctorius* was

effective in forming mycorrhizae on seedlings one month following seed germination. Numerous sporophores of *P. tinctorius* were produced in these plots during August to October. After the seedlings became dormant in December they were lifted and evaluated. The mycelial inoculum formed the distinctive gold-yellow ectomycorrhizae of *Pisolithus* on 92 per cent of the feeder roots which induced more than a 100 per cent increase in total dry weights of seedlings over the controls. The control seedlings had 45 per cent of their feeder roots colonized with the cream-brown ectomycorrhizae characteristic of *Thelephora terrestris*. The visual estimate of the amount of mycorrhizae formed by each of the fungi was confirmed by reisolation of the respective fungi from the mycorrhizae and also by a fluorescent antibody technique developed for each of the fungi (Schmidt, Biesbrock, Bohlool, and Marx 1974). From these experiments it was found that the mycorrhizae formed by these two fungi could be visually assessed accurately on intact seedlings with the unaided eye. It should be pointed out, however, that only one other ectomycorrhizal type was present on seedlings. It occurred infrequently and was a pure white, coralloid type, easily distinguished from either *P. tinctorius* or *T. terrestris* mycorrhiza.

Survival of mycelial inoculum. A major concern in the use of pure mycelial cultures of ectomycorrhizal fungi expressed by other researchers (Moser 1963; Takacs 1967) is survival of inoculum in soil. Mycelial inoculum of *P. tinctorius* will survive in soil under a variety of conditions. During early months of the 1972 study (Marx and Bryan 1975), particles of the inoculum were removed periodically from the soil. Mycelium of *P. tinctorius* with good structural integrity was observed microscopically to be abundant in the laminated structure of the vermiculite particles. Mycelium within the leached vermiculite particles is apparently protected from environmental extremes and extensive saprophytic colonization. Residual inoculum will also survive in soil after overwintering. After the seedlings were removed from the microplots in December, soil which was initially infested with the mycelial cultures of *P. tinctorius* was left fallow until the following spring. Soil temperatures dropped to −7 °C on several occasions during the winter. In April soil in the plots was mixed with freshly fumigated soil at different ratios, seeded with *P. taeda*, and in December the seedlings were lifted and evaluated. Seedlings in non-diluted soil formed mycorrhizae with *P. tinctorius* on 75 per cent of the feeder roots. In the 1:1, 1:2.5, 1:5, and 1:10 dilutions, 60, 35, 25, and 15 per cent of the feeder roots, respectively, were ectomycorrhizal with this fungus. *Thelephora* mycorrhizae also occurred on these seedlings.

Another test of the persistence of vermiculite-peat moss inoculum of *P. tinctorius* was done in a pot study in the greenhouse (Marx, unpublished data). Leached inoculum was added to fumigated soil in a 1:8 ratio and incubated without a tree host at soil temperatures of 15, 20, 25, and 30 °C. After two weeks, one, two, and three months at the different temperatures, the infested soil was planted with seed of *P. taeda* and then incubated at 25 °C. Six months later the seedlings were evaluated. The seedlings in soil originally incubated for three months at 30 °C without a tree host had nearly as many ectomycorrhizae formed by *P. tinctorius* as seedlings in soil originally incubated without a host for only two weeks at 15 °C. We concluded from these studies that leached inoculum of *P. tinctorius* can survive in soil under a variety of conditions.

Nursery tests with mycelial inoculum. Following the test in the microplots (Marx and Bryan 1975), leached vermiculite-peat moss inoculum of *P. tinctorius* was introduced into fumigated soil at state nurseries in Georgia, Florida, and North Carolina (Marx *et al.* 1976). The volume of leached inoculum used in each nursery was 2.8 l/m^2 of soil surface. It was broadcast onto the soil and immediately mixed thoroughly with hand tools into the upper 10 to 12 cm of soil.

In the Georgia nursery, ineffective soil fumigation apparently precluded successful colonization of *P. taeda, P. clausa*, or *P. virginiana* seedlings by the introduced inoculum. Problems with soil fumigation were evident by the very high levels of plant parasitic nematodes, phycomycetous root pathogens, and diseased pine seedlings detected at the end of the growing season. Also, the appearance of *T. terrestris* sporophores and mycorrhizae on seedlings as early as the second month after seeding suggested that the residual inoculum of this fungus from the previous year was not seriously affected by the fumigation.

Soil fumigation in the other two nurseries was effective. In the Florida nursery, seedlings of *P. taeda, P. clausa,* and *P. elliottii* var. *elliottii* had abundant *Pisolithus* mycorrhizae as early as six weeks after seed germination. Control seedlings of all pines also had a few mycorrhizae formed by naturally occurring fungi at this time. Numerous sporophores of *P. tinctorius* were produced in all plots inoculated with mycelial cultures by August or September. Considerably more sporophores were produced in *P. taeda* and *P. elliottii* plots than in *P. clausa* plots. Seedlings were lifted and evaluated eight months after study installation. Although *Pisolithus* mycorrhizae were formed in abundance, differences in seedling growth and total mycorrhizal development were not detected. Inoculated seedlings of *P. taeda* had 72 per cent mycorrhizal development of which *P. tinc-*

torius formed about one-half. Control seedlings of *P. taeda* had 66 per cent mycorrhizal development, all of which were formed by naturally occurring fungi. On inoculated seedlings of *P. elliottii* var. *elliottii*, *P. tinctorius* formed about eight-tenths of the total mycorrhizal development of 82 per cent. Control seedlings had 73 per cent development by other fungi. *Pisolithus tinctorius* formed over half of the 40 per cent mycorrhizal development on seedlings of *P. clausa*. Control seedlings of *P. clausa* only formed mycorrhizae with naturally occurring fungi on 21 per cent of the feeder roots. There was a positive relationship between the number of sporophores and the amount of mycorrhizae produced by *P. tinctorius* on the different pine species. The fewest number of sporophores were produced in plots of *P. clausa* seedlings which also had the fewest roots colonized by *P. tinctorius*.

In the North Carolina study, mycorrhizal development early in the season on seedlings in inoculated and control plots and the late summer development of sporophores of *P. tinctorius* were similar to that observed in the Florida study. However, in the North Carolina study, stimulation of seedling growth (total fresh weights) by *Pisolithus* mycorrhizae was 140 per cent on seedlings of *P. taeda* and about 100 per cent on seedlings of *P. virginiana* and *P. strobus*. Total mycorrhizal development on all pine species was also increased significantly by mycelial inoculation with *P. tinctorius*. Inoculated seedlings of *P. taeda* had a total of 64 per cent mycorrhizal development, with over nine-tenths formed by *Pisolithus*. Control seedlings had 50 per cent mycorrhizal development by naturally occurring fungi. The inoculated seedlings of *P. virginiana* had a total development of 72 per cent with two-thirds formed by *P. tinctorius*. Control seedlings had 47 per cent of their feeder roots ectomycorrhizal. Seedlings of *P. strobus* had a total development of 47 per cent with about three-quarters formed by *Pisolithus*. Control seedlings only formed 15 per cent mycorrhizae with other fungi. This study showed that following proper soil fumigation, leached vermiculite-peat moss inoculum of *P. tinctorius* can be introduced into soil of conventional tree nurseries and form abundant mycorrhizae on roots of southern pines. The field performance of these seedlings after outplanting on different routine reforestation sites will be discussed later.

Our next nursery research (Marx and Artman 1978) involved comparing the response of *P. taeda* seedlings to inoculation with leached vermiculite-peat moss cultures of *P. tinctorius* and *T. terrestris*. Studies were installed in fumigated soil in two state nurseries in Virginia, one in the coastal plain and the other in the mountains (elevation 580 m). Inoculum of each fungus was applied at a rate of 1.08 l/m^2 of soil surface and mixed thoroughly into the soil. Control

plots received the same amount of vermiculite. This rate of inoculum was used because results from an inoculum density study, to be discussed later, indicated that it is as effective as higher rates. After seeding in April 1975, the seedlings were grown for seven months, lifted, and evaluated.

Only two morphological types of ectomycorrhizae were observed on seedlings in both nursery tests. One type, formed by *T. terrestris*, was observed almost exclusively in *T. terrestris* inoculated plots and the control plots. The other type was formed by *P. tinctorius* and it was observed only in *P. tinctorius* inoculated plots. *Pisolithus* formed nearly nine-tenths of all the mycorrhizae (75 per cent) in both the coastal plain and the mountain nursery. In both nurseries non-inoculated control seedlings had about 46 per cent mycorrhizal development. There were four to five times more sporophores of *T. terrestris* in the plots inoculated with *T. terrestris* in both nurseries than in control plots. Sporophore production by *P. tinctorius* was not recorded, but it was observed in all *Pisolithus* plots. Seedlings from *Pisolithus* and *Thelephora* inoculated plots had 57 and 31 per cent greater fresh weights in the coastal plain nursery and 40 and 20 per cent greater fresh weights in the mountain nursery, respectively, than the controls. Even though *P. tinctorius* and *T. terrestris* formed the same quantity of mycorrhizae on the seedlings, those with *Pisolithus* mycorrhizae were significantly heavier in both nurseries than those inoculated with *T. terrestris*. This indicated that *P. tinctorius*, even though ecologically adapted to soils of low fertility and other adverse conditions, is probably more efficient than *T. terrestris* in maximizing nutrient absorption from soil. It may be that mycorrhizal fungi adapted to poor soils make more efficient use of available nutrients than other fungal symbionts adapted to better soils.

In North Carolina, Krugner (1976) examined the interaction of soil fertility with these two fungi in closer detail on *P. taeda* in a microplot study. Using leached vermiculite-peat moss inoculum of each fungus, fumigated soil was infested with either *P. tinctorius*, *T. terrestris*, an equal mixture of inoculum of both fungi, or autoclaved inoculum of both fungi (control). The inoculum was standardized at 2 l/m^2 of soil surface for all treatments and mechanically mixed into the upper 10 to 12 cm of soil. Fertility treatments of N at 145 kg/ha, NPK at 145, 50, and 100 kg/ha, respectively, and no added fertilizer were imposed on the fungal and control treatments. After eight months the seedlings were lifted and evaluated. Inoculation of soil with either fungus alone or in mixture did not markedly affect seedling growth. Independent of the fungi, both fertilizer treatments significantly increased seedling growth in comparison to

non-fertilized seedlings. Both fertility treatments stimulated the development of *Pisolithus* mycorrhizae whether *P. tinctorius* was added to soil alone or in mixture with *T. terrestris*. *Pisolithus* formed about one-fifth of the total mycorrhizal development of 64 per cent in non-fertilized soil, about one-half of the total development of 80 per cent in the nitrogen-treatment, and about two-thirds of the total mycorrhizal development of 80 per cent in complete NPK treatment. Seedlings in *T. terrestris* and control plots had between 55 per cent and 62 per cent mycorrhizal development in all fertility treatments, including the non-fertilized controls. Krugner concluded that *T. terrestris* did not compete well with *P. tinctorius* for seedling roots under conditions of abundant nutrient availability. He suggested that the inoculum of *T. terrestris* may not have been as vigorous as that of *P. tinctorius*. This latter point is undoubtedly a factor to consider in work of this type. However, *P. tinctorius* may simply be able to compete for roots better than *T. terrestris* in soils with good fertility, at least for the first growing season. Different results may be obtained in soil of higher fertility or in nursery studies of longer duration.

In the previous microplot and tree nursery studies natural recolonization of the fumigated soil by wind-disseminated spores of ectomycorrhizal fungi indigenous to the areas was very rapid and efficient. Usually within a few weeks after seed germination, mycorrhizae were formed on seedlings in previously non-inoculated soil by naturally occurring fungi. Competition existed, therefore, between the natural spore inoculum of the indigenous fungi and the inoculum of the artificially introduced fungi very early in the growing season. In 1974 we were able to examine the significance of soil inoculation with pure mycelial cultures to seedling growth and mycorrhizal development in a new tree nursery having minimal competition from native ectomycorrhizal fungi (Marx *et al.* 1978). The nursery, located in south-eastern Oklahoma, was established on former pasture land in an area surrounded by only a few scattered ectomycorrhizal trees. In 1974 the first crop of *Pinus taeda* seedlings was grown in non-fumigated soil. Recommended rates of fertilizer and pesticides were applied during the growing season. By mid-July the seedlings were stunted and chlorotic; less than 10 per cent of the seedlings had a trace of mycorrhizae. By mid-August thousands of seedlings were dying each week. More fertilizer and pesticides were added but seedling mortality continued. In January 1975, the nearly seven million seedlings were lifted and evaluated. Only 4 per cent had acceptable stem diameters (greater than 3 mm), none met the 15 cm height requirement for planting out, and very few seedlings had mycorrhizae. The nursery managers concluded that the poor growth of seedlings resulted from an insufficient quantity of mycorrhizae.

In April of 1975, a comprehensive study was installed in a new section of this nursery using pure mycelial cultures of *P. tinctorius, T. terrestris,* and *C. graniforme* in both fumigated and non-fumigated soil. Vermiculite-peat moss cultures of *P. tinctorius* and *T. terrestris* were grown for three months and the total volume of each culture vessel was leached and used as inoculum. However, the slower growing *C. graniforme* did not colonize all the mixture in three months; therefore only that part of the substrate with obvious mycelium of *C. graniforme* was leached and used as inoculum. Mycelial inoculum of all fungi was broadcast at a rate of 1.08 l/m^2 of soil surface and mixed into the upper 10 to 12 cm of soil. Control plots received the same rate of vermiculite. *P. taeda* was seeded in April, and during the growing season all seedlings received the same amount of fertilizer and water.

Approximately six weeks after seeding, ectomycorrhizae of *P. tinctorius* and *T. terrestris* were observed on seedlings in their respective plots in both fumigated and non-fumigated soil. Seedlings in other plots had only a few mycorrhizae at this time. By mid-August sporophores of both fungi were detected in their respective plots in fumigated and non-fumigated soil. Seedlings in these plots were vigorous and were nearly twice as large as seedlings in other plots. Mid-season examination of these vigorously growing seedlings showed they had 35 to 40 per cent of their feeder roots mycorrhizal with the respective fungi. Only a few black mycorrhizae of *C. graniforme* were observed on seedlings in the *C. graniforme* plots at this time. By early October, seedlings in the control non-fumigated plots and the fumigated and non-fumigated plots of *C. graniforme* began to grow at normal rates. Concurrent with this new growth was the appearance of *Thelephora* mycorrhizae on the control seedlings and *Thelephora* and *C. graniforme* mycorrhizae on the seedlings in the *C. graniforme* plots. Non-inoculated seedlings in fumigated soil were still stunted and had few mycorrhizae.

In December the 54 000 seedlings in this study were lifted and representative ones evaluated. In fumigated soil, the number of plantable seedlings (greater than 12.5 cm in height and 3 mm in root collar diameter) was increased by 155 per cent over the controls with *T. terrestris*, 140 per cent with *P. tinctorius*, and 77 per cent with *C. graniforme*. The former two fungi also increased the number of plantable seedlings in non-fumigated soil over the non-inoculated controls. Non-fumigated control plots had over twice as many plantable seedlings as fumigated control plots. None of the fungal treatments significantly increased seedling size in non-fumigated soil. However, in fumigated soil *T. terrestris* and *P. tinctorius* increased total fresh weights of plantable seedlings by 125 per cent and *C. grani-*

forme by 24 per cent. Mycorrhizal development was also affected by soil fumigation. In fumigated plots *Pisolithus* mycorrhizae accounted for eight-tenths of the total 60 per cent development. In non-fumigated soil, total development was about 55 per cent with *Pisolithus* forming over two-thirds of these. Since *Thelephora* occurred naturally in this nursery, it was difficult to make accurate assessments of the value of inoculation with this fungus. Naturally occurring fungi other than *T. terrestris* were less frequent on seedlings on fumigated soil inoculated with *Thelephora* than in non-fumigated soil, but total mycorrhizal development was significantly greater in fumigated (59 per cent) than in non-fumigated (48 per cent) soil. There was just as much naturally occurring *Cenococcum* mycorrhizae on seedlings in the non-fumigated control plots as in the fumigated soil inoculated with *Cenococcum*. *Cenococcum* mycorrhizae accounted for about one-quarter of the mycorrhizae in fumigated inoculated plots and accounted for one-eighth of the mycorrhizae in the non-fumigated inoculated plots. All of the above mycorrhizal assessments of specific fungi were confirmed by surface sterilizing the mycorrhizae and reisolating the fungi on agar medium.

The results of this study revealed several salient points. Mycorrhizal development must occur early in the growing season in order to improve the numbers of plantable seedlings of *P. taeda* and their size. Pure mycelial cultures of specific fungi can be used to correct the erratic occurrence or deficiency of mycorrhizae in both fumigated and non-fumigated nursery soil in a geographic area where few symbiotic fungi occur naturally. Soil fumigation obviously reduces populations of indigenous symbiotic fungi and other micro-organisms, improving the success of artificial soil inoculations with pure mycelial cultures of *P. tinctorius* and *T. terrestris*. Lastly, it appears that *C. graniforme*, owing to its inherently slow growth rate and its adaptation to drought-prone soils, will not effectively colonize roots of pine seedlings in irrigated nursery soils. Perhaps with maintenance of less soil moisture in nurseries where soil colonization by other symbiotic fungi is slow, this fungal symbiont may be effectively maintained on seedling roots.

Rate of mycelial inoculum. During our research with *P. tinctorius* it became apparent that we did not know the least amount of inoculum needed to successfully infest soil and form ectomycorrhizae on seedlings. In many instances the amount of inoculum used in our early tests was dictated by the amount of inoculum available for use at the time. To examine this problem, a study was installed in a tree nursery in Mississippi (Marx, unpublished data). This nursery has been producing good quality pine seedlings for over 25 years that are

usually heavily mycorrhizal with *T. terrestris* and other fungi. *Pisolithus* has also been observed in this nursery. In the spring of 1976, leached vermiculite-peat moss inoculum of *P. tinctorius* was broadcast on fumigated soil at rates of 2.80, 2.16, 1.62, 1.08, and 0.5 l/m^2 of soil surface. Two control treatments were installed; one received 2.8 l/m^2 rate of leached autoclaved inoculum and the other received no inoculum. These were used to delineate any possible physical or chemical effects of the inoculum to the soil and seedling growth. Seeds of *Pinus palustris* and *P. echinata* were planted and the plots mulched. In December, approximately 26 000 seedlings of each pine species were lifted and representative seedlings were evaluated.

Inoculation with *P. tinctorius* at any rate significantly increased total mycorrhizal development from 23 per cent (mean of both control groups) to 34 to 43 per cent on *P. palustris* seedlings. *Pisolithus* mycorrhizae, regardless of the inoculum rate, accounted for one-third of the total development. Significant increases in seedling growth and the number of plantable seedlings were associated with all inoculation treatments. Seedlings of *P. echinata* were also stimulated regardless of inoculum rate. On this species, *Pisolithus* mycorrhizae at the four highest inoculum rates, accounted for about one-third of the total development, but it formed only about one-quarter of all mycorrhizae at the 0.54 l/m^2 rate. *Thelephora terrestris* formed most of the other mycorrhizae. A comparison of seedlings and soil from the two different control treatments showed that the leached inoculum did not affect seedling growth or change chemical (major nutrients) or physical (cation exchange capacity) conditions of the soil. The 1.08 litres per m^2 of soil surface rate was the least amount that could effectively be used for maximum mycorrhizal development in this nursery. This nursery has one of the most rapid colonizations of fumigated soil by *T. terrestris* of any nursery in which we have worked. Therefore, this 1.08 l/m^2 rate may be even more effective in nurseries having a lesser degree of early competition from other fungi. We are currently recommending this rate for purposes of experimentation in properly fumigated nursery soils.

Drying of mycelial inoculum. The weight and physical nature of pure mycelial inoculum that had been used up to this time caused certain problems. After leaching, the vermiculite-peat moss inoculum had a very high weight to volume index and physically resembled a sticky paste. The inoculum was also very heavy to transport and quite difficult to spread and mix into the soil. A study was conducted to determine the feasibility of drying the inoculum of *P. tinctorius* in order to eliminate these problems (Marx, unpublished data). Vermiculite-peat moss inoculum was leached in tap water, squeezed

to remove excess water, placed 2 cm deep in an aluminium tray and dried to 12 per cent moisture at 28–30 °C for 56 hours in a forced-air oven. In order to ascertain the effects of drying on the efficiency of the inoculum, it was added to soil at different rates. Leached but non-dried inoculum was used in identical fashion for a further comparison. Inoculum was broadcast on fumigated soil in microplots at our nursery in Athens at rates of 2.16, 1.08, 0.54, and 0.27 l/m^2 of soil surface and mixed 10 cm deep into the soil. Control soil received vermiculite at the highest rate. Seed of *P. taeda* were planted in April 1976 and nearly 8000 seedlings were lifted and evaluated the following December. Dried inoculum was as good as, if not better than, non-dried inoculum for development of *P. tinctorius* mycorrhizae. An average of the three highest rates showed that *Pisolithus* from non-dried inoculum formed less than one-third of the 73 per cent total development while dried inoculum formed nearly half of the mycorrhizae. The lowest rate (0.27 l/m^2) of both inoculum formulations formed less than half the amount of *Pisolithus* as the higher rates. Since procedures for soil fumigation are more efficient at our nursery facility than those employed in conventional nurseries, we obtained greater effectiveness at lower rates of both inoculum formulations in this study than obtained from the inoculum rate study in the Mississippi nursery. One reason for the greater effectiveness of the dried inoculum is that it mixes more homogeneously in soil than the paste-like, non-dried inoculum. Removal of excess water from leached inoculum reduced the volume by one-third; drying reduced it further by nearly a third. An initial 3 litre volume of inoculum from culture vessels is reduced to 1.2 to 1.4 litres of usable inoculum after it is leached and dried.

Storage of mycelial inoculum. Severe limitations would be placed on the broad scale use of this type of inoculum of any fungus if the inoculum could not survive reasonable lengths of storage. There would be few cases where transport of inoculum from the laboratory to the nursery did not entail a period of storage under various temperature conditions. The following study (Marx, unpublished data) investigated the influence of length of storage at different temperatures on the effectiveness of dried and non-dried inoculum of *P. tinctorius*. Inoculum was prepared, as previously described, and 15 ml volumes were stored in test tubes at 5, 23, and 30 °C. At weekly intervals sets of tubes were removed from the incubators. The inoculum was mixed at a 1:8 ratio with fumigated soil and placed in small pots in the mycorrhizal fungus-free growth room. Seed of *P. taeda* were planted and seedlings were evaluated after four months. Non-stored, dried inoculum formed 50 per cent *Pisolithus* mycorrhizae

and the non-dried inoculum formed 57 per cent. This proved initial viability of inoculum. After the first week of storage viability dropped to 48 per cent mycorrhizal development for non-dried and 41 per cent for dried inoculum. This level of viability was maintained for the next seven to nine weeks of storage for inoculum incubated at 5 and 23 °C and for five to seven weeks at 30 °C. Viability decreased significantly after longer periods. The fact that leached and dried inoculum of *P. tinctorius* can be stored for up to nine weeks at refrigeration temperatures and for at least five weeks at warmer temperatures indicates that it is quite durable and should withstand reasonable storage and transportation conditions.

It is apparent from the discussions of research carried out by scientists in various parts of the world that the artificial introduction of specific fungi into nurseries and containers is biologically feasible. Published reports indicate that inoculation programmes developed in Austria and Argentina are on a quasi-operational level for practical application. In the United States, a test programme is currently underway to determine the feasibility of producing inoculum of *P. tinctorius* for commercial uses. In the spring of 1977, Abbott Laboratories, Long Grove, Illinois, produced a dried, vermiculite-peat moss inoculum of *P. tinctorius* which we tested in 19 identical nursery experiments in 15 states of the South-East, South, and South-West. Seven different species of *Pinus* and *Quercus rubra* were involved. In each experiment different rates (1.62, 1.08, and 0.54 l/m^2 of soil surface) of the Abbott-produced inoculum and one rate (1.08 l/m^2) of dried inoculum produced in our laboratory were compared. This inoculum was further evaluated in seedling container programmes in five different states involving eight species of *Pinus, Pseudotsuga menziesii,* and *Tsuga heterophylla.* From September 1977 to March 1978 over 150 000 seedlings were lifted and representative seedlings were evaluated in Athens. Although the results were erratic and somewhat inconsistent, they showed that the dried, vermiculite-peat moss inoculum of *P. tinctorius* can be produced in large volumes in industrial fermentors and is functional in forming mycorrizae on seedlings. In the spring of 1978 our tests were expanded to include the entire United States using an improved inoculum production method. 33 bare-root and 11 container nursery tests are currently underway. These tests involve all major ectomycorrhizal tree species grown in the United States. Studies will be terminated in the bare-root nurseries after one, two, or three years depending on the tree species under test. We are very optimistic about the biological value of this commercially produced inoculum. If this product form of *P. tinctorius* inoculum proves to be functional, Abbott Laboratories has the fermentor capacity to potentially

produce hundreds of thousands of litres for application in world forestry.

Fungus selection criteria and maintenance of pure cultures

The most important first step in any nursery inoculation programme is the selection of the fungi (Bowen 1965; Marx 1977a; Mikola 1973; Moser 1963; Trappe 1977). The physiological differences that exist between mycorrhizal fungi can be used as criteria for their selection. The importance of each of the following criteria will vary according to the needs of the different inoculation programmes in different locations. Therefore, criteria will not be ranked in this discussion.

Host specificity

One criterion is host specificity. The consistent association of certain fungi for only a few specific tree hosts is well documented in the literature. Many other fungi are associated with a great number of different tree hosts (Marx 1977b; Stevens 1974; Trappe 1962). It is imperative, therefore, that the candidate fungi exhibit the physiological capacity to form mycorrhizae on the desired hosts. There is another aspect to this criteria, however. It is not sufficient to simply select a fungal species and then obtain an isolate for testing. Several isolates from different tree hosts and geographic regions should be used. This point has been stressed by Moser (1958c) and demonstrated by Theodorou and Bowen (1970) with isolates of *Rhizopogon luteolus*. We have obtained isolates of *P. tinctorius* from different species of oaks and compared them with isolates from pine in the mycorrhizal fungus-free room and in the microplot nursery on *Quercus rubra* seedlings (Marx, unpublished data). The pine isolates formed abundant mycorrhizae in the growth room and nursery. Some oak isolates formed a few mycorrhizae; some isolates did not form mycorrhizae at all. All isolates were similar in age and had comparable pigmentation and rates of vegetative growth in agar medium.

Growth in pure culture

Another criterion is the ability of the selected fungi to grow in pure culture; many ectomycorrhizal fungi will not. A variety of culture media (Moser 1958b; Stevens 1974; Trappe 1962) and methods of isolation (Palmer 1971) can be used to obtain pure cultures of the selected fungi. Ideally, the fungi should be able to grow rapidly (Moser 1959). Once cultures of the selected fungus have been obtained they must be maintained in a viable condition. Takacs (1967) recommends subculturing the stock cultures of the fungi every 60 days in order to retain vigour. Moser (1958b) stressed the need to

subculture every two to five weeks, depending on fungus species, especially those to be used in current inoculation programmes. If the cultures are not subcultured frequently they exhibit poor growth and loss of pigment. He recommends growing declining cultures on a different medium to rejuvenate them. We found that continuous culturing of certain fungi on agar media for several years frequently decreased mycelial growth rate and the capacity to form mycorrhizae on pine. Changes in adaptive enzyme systems during continuous vegetative growth on synthetic medium probably accounts for this loss of ability to symbiotically infect the host roots. Mycelial agar discs cut from plate cultures and stored in sterile distilled water at 5 °C can be held for up to three years without loss of these physiological traits (Marx and Daniel 1976). The technique does not work for all fungi but is worthy of testing. The storage of cultures in a dormant physiological state should reduce, if not eliminate, shifts in adaptive enzyme systems. A certain amount of caution, therefore, must be used in evaluating fungi maintained in continuously growing, pure cultures for extended periods of time. We had isolates of *P. tinctorius* grown in continuous culture for 15 years that were still highly pigmented and grew at rates comparable to that achieved shortly after their isolation from sporophores. In 1974 these isolates lost their capacity to form mycorrhizae on pine. One of these (isolate 29) had been used successfully by us in several earlier studies (Marx, Bryan, and Davey 1970) since its original isolation. We have found, however, that our best isolates of *P. tinctorius* are those that are cycled back through their host every year or two and then reisolated from sporophores or directly from mycorrhizae. Our main isolate of *P. tinctorius* currently under test with Abbott Laboratories was first isolated in 1967 from a sporophore under a mature *P. taeda* growing in Georgia. This isolate has been rejuvenated by cycling it through pine hosts every one or two years. Today, it grows faster and forms more mycorrhizae on pine and oak than it did in 1967. It also has formed mycorrhizae on a variety of host species that other recently cultured isolates have not. A parent culture of this isolate maintained in continuous culture will form few mycorrhizae at this time.

If spores of a selected fungus are to be used for inoculum, then the ability of this fungus to grow well in pure culture is of little importance. However, growth in pure culture may be useful if it is to be reisolated from mycorrhiza to confirm its identity.

Once the growth potential of a fungus has been confirmed it is important to confirm its capacity to withstand physical manipulation (leaching, drying, soil incorporation, colonization by saprophytes, etc.). Producing large quantities of inoculum of a fungus is of little value if the fungus cannot survive the rigours of various manipulations

essential to inoculation of soil. Certain fungi grow readily in vermiculite-peat moss medium but cannot survive the leaching procedure or soil inoculation. If we had not studied the colonization of non-leached mycelial inoculum of *P. tinctorius* by various saprophytic micro-organisms and rectified the problem by leaching, we could have easily concluded that *P. tinctorius* was not a good fungal candidate for any inoculation programme.

Fungus adaptability

Another criterion is the adaptation of the selected fungus to the major type of site on which the seedlings are to be outplanted. Of equal importance is the ability of the fungus to survive and grow under cultural conditions used in nurseries. According to Trappe (1977), the ecological adaptability of an ectomycorrhizal fungus hinges on the metabolic pathways it has evolved to contend with environmental variation. Extremes of soil and climatic factors, antagonism from other soil organisms, pesticide application, physical disruption of mycelium from nursery operation, and the abrupt adjustment from a fertilized and irrigated nursery soil to an uncultivated planting site with all of its stresses are only a few of the environmental variations to which the selected fungi must adapt.

The effect of temperature on different species and ecotypes of ectomycorrhizal fungi is perhaps the most widely researched environmental factor. Upper and lower temperature limits of the candidate fungi should be determined. Moser (1958d) studied the ability of fungi to survive long periods (up to four months) of freezing (–12 °C) and to grow at low temperatures (0–5 °C). He found that high elevation ecotypes of *Suillus variegatus* were not damaged after freezing for two months, but valley ecotypes were killed after freezing for only five days. Although not as striking, similar results were reported with *S. tridentinus, S. plorans,* and *Gomphidius rutilus.* In low temperature growth studies, none of the species of *Amanita* grew at 5 or 0 °C. An interesting observation was that certain species and ecotypes which survived freezing for extended periods did not grow at low temperatures. Generally, he found that mountain ecotypes and species had much lower temperature optima than lowland ones. Even after several years in pure culture at 20 to 23 °C, the low temperature fungi still maintained optima near 15 °C. *Pisolithus tinctorius* not only survives and grows well at unusually high temperatures, but also it grows at 7 °C and survives in frozen soil (Marx, Bryan, and Davey 1970, Marx and Bryan 1971, 1975). High temperature tolerance makes *P. tinctorius* an excellent candidate for testing in the tropics (Momoh and Gbadegesin 1975). *Rhizopogon luteolus* apparently is not suitable for inoculation programmes because of its inability to

survive or grow at the high soil temperatures common to this area (Momoh 1973).

Reaction of the candidate fungi to soil moisture, organic matter, and pH are also important traits to consider. *Cenococcum graniforme* is not only drought tolerant but forms mycorrhizae in natural soils ranging in pH from 3.4 to 7.5 (Trappe 1964). We have observed *Pisolithus* mycorrhizae on pine in drought-prone coal spoils ranging in pH from 2.6 to as high as 8.4. Trappe (1977) has observed several species of fungi which form ectomycorrhizae in well-rotted conifer logs with a pH of 4.0 or lower in the Pacific North-west of the United States. Levisohn (1965) observed in England that *Suillus bovinus*, an excellent mycorrhizal fungus on spruce, naturally occurs in nursery soils containing abundant organic matter. Unfortunately the fungus disappears from the roots of spruce planted on sites having low organic matter. Its potential value in inoculation programmes would appear to be restricted to sites with high levels of organic matter.

Value of hyphal strands

Another criterion by which candidate fungi should be evaluated is their capacity to form hyphal strands in pure cultures and in soil. Bowen (1973) showed that nutrient uptake, especially phosphorus, is greater in fungi that produce hyphal strands. In Australia, one of the initial criteria for selection of fungi is their ability to produce hyphal strands under a wide range of conditions. Although research data is lacking we believe that the abundant hyphal strands produced by *P. tinctorius* not only enhance nutrient absorption, but increase its survival potential under adverse conditions. Yellow-gold hyphal strands of *P. tinctorius*, easily visible to the naked eye, have been traced through highly toxic and hot coal spoils as far as 4 m from seedlings to sporophores by Schramm (1966) and others (Marx 1977a). On an exposed borrow pit in South Carolina we traced hyphal strands of *P. tinctorius* over 3 m from mycorrhizal roots of *P. palustris* to sporophores.

Aggressiveness of fungus

Another extremely important criterion is the aggressiveness of the candidate fungus to feeder roots. The fungus should have the capacity to form abundant mycorrhizae as soon as feeder roots are formed. It must be able to maintain superiority over naturally occurring fungi in the nursery. Aggressiveness is best evaluated by making quantitative assessments on the amount of mycorrhizae formed by the introduced fungus at different intervals of time. Quantitative assessments are the only valid parameter which can be used to judge the effectiveness of inoculations. We have found that maximum

benefit of *Pisolithus* mycorrhizae is achieved on pine seedlings when at least two-thirds of all the mycorrhizae on the seedlings are formed by *P. tinctorius*.

Field performance of seedlings with specific ectomycorrhizae

The ultimate proof of the value of inoculation of bare-root or container grown nursery seedlings with specific fungi is their performance under diverse field conditions. Meaningful conclusions can only be obtained from properly designed, installed, and maintained field experiments which include periodic tree measurements and mycorrhizal assessments conducted over several years. Only limited field data of this type is available in the literature. Moser (1963) reported that spruce seedlings with mycorrhizae formed by *Phlegmacium glaucopus* survived and grew better than comparable non-mycorrhizal seedlings on a 2100 m altitude forest site in Austria. In another test, four-year-old nursery grown seedlings of *Pinus cembra* with few mycorrhizae were planted on a 2100 m altitude site. These seedlings were inoculated (apparently at planting time) with an equal mixture of pure mycelial inoculum of *Suillus plorans, S. placidus, Paxillus involutus,* and *Amanita muscaria*, a mixture of mycelial inoculum of these four mycorrhizal fungi contaminated with *Penicillium, Mucor*, and bacteria, or no inoculum. After three years the mixed inoculum (either pure or half-pure) of the symbiotic fungi stimulated height growth and increased the number of healthy seedlings by 65 per cent over the non-inoculated controls. Half-pure inoculum of the fungi was only slightly less effective in stimulating seedling survival and growth than the pure mycelial mixture. An assessment of mycorrhizal development was not reported in this study.

Puerto Rico

In Puerto Rico in 1965, Vozzo and Hacskaylo (1971) outplanted seedlings of *Pinus caribaea* from one of the container nursery experiments on a sandy loam site. Unfortunately, damage to seedlings by vandals and cattle shortly after planting resulted in study termination after only six months. Results showed, however, that mycorrhizae formed by *Suillus cothurnatus, Rhizopogon roseolus, Corticium bicolor*, and by unidentified fungi in natural soil inoculum stimulated height growth of the seedlings over both fertilized and non-fertilized, non-mycorrhizal seedlings. Regardless of fertility, non-mycorrhizal seedlings were chlorotic and stunted. At the time of planting, 75 per cent of the seedlings inoculated with pure mycelial cultures and 95 per cent of the seedlings inoculated with the natural inoculum had ectomycorrhizae. Apparently the degree of development on individual

seedlings was not determined. Their results indicated that *S. cothurnatus*, *R. roseolus*, and *C. bicolor* in pure mycelial inoculum can be used in Puerto Rico for the establishment of *P. caribaea.*

Australia

In Australia, Theodorou and Bowen (1970) installed two field experiments with *Pinus radiata* seedlings. In the first test, one-week-old seedlings were transplanted into fumigated potting mixture contained in small wooden veneer tubes. Each tube contained a 10 g layer (3 cm deep in tubes) of pure mycelial inoculum of either *Suillus granulatus*, *S. luteus*, or two isolates of *Rhizopogon luteolus*. Sterile medium was added to control tubes. The seedlings were grown for four months in a greenhouse and then hardened off for an additional three months in the open prior to outplanting. The seedlings were planted in 1966 on a loamy soil field site some 200 m from an established stand of *P. radiata.* At planting, all inoculated seedlings were 7 cm tall and had about 25 per cent mycorrhizal development. Control seedlings were 6 cm tall with only a trace of mycorrhizae. The field design was a randomized design with three blocks. A buffer row surrounded each plot within each block. Significant differences in height occurred as early as six months after planting. Seedlings with *S. granulatus* or *R. luteolus* mycorrhizae were about 46 per cent taller (13.9 cm) than control seedlings (9.5 cm). Seedlings with *S. granulatus* were also noticeably greener than seedlings of other treatments. All seedlings had a healthy green colour after 28 months. Following a summer drought, nearly three times more control seedlings had died (13 per cent) than did those inoculated with *S. granulatus* or *R. luteolus* (3-5 per cent). 20 per cent of the seedlings with *S. luteus* died during the drought. All inoculated seedlings were significantly taller than controls after eight months. After 32 months, the rate of height growth of seedlings with *S. granulatus* mycorrhizae was significantly greater than control seedlings. At 36 months the rate of growth was similar, but differences in height that developed earlier were still evident. Root evaluations revealed that differences in growth due to the different fungi were related to the degree of ectomycorrhizal development. *Suillus granulatus* formed significantly more mycorrhizae (81 per cent) during the 36 months than did the two *R. luteolus* isolates (78 per cent), *S. luteus* (68 per cent), or the control seedlings (65 per cent). White mycorrhizae typical of those produced by the test fungi dominated inoculated seedling roots and a brown-type was observed on the control seedlings.

In their second field test Theodorou and Bowen (1970) grew *Pinus radiata* seedlings as before, except *S. granulatus, S. luteus, R. luteolus*, and an unidentified isolate obtained from mycorrhizae of nursery

seedlings were used. At planting all seedlings were 6 to 7 cm tall. Inoculated seedlings had 16 to 23 per cent mycorrhizal development and control seedlings had none. These seedlings were outplanted 900 m from an established *P. radiata* stand. After 23 months, there were no significant differences in seedling heights or development of mycorrhizae between treatments. The control seedlings had a similar amount of mycorrhizae (76 per cent) to the inoculated seedlings (69 to 82 per cent). A white ectomycorrhizal type which apparently occurred naturally on this site was observed early on inoculated and control seedlings. This natural colonization of roots of control seedlings obviously minimized the effect of inoculations. The authors stressed the need for larger field plots, more extensive buffers between plots, and test sites which do not contain ectomycorrhizal fungi of *P. radiata* for future studies. They feel these conditions are essential to valid testing of the significance of the various fungi. In spite of problems in the second study, their results suggested that pure mycelial cultures of *S. granulatus* and *R. luteolus* can be used to form abundant mycorrhizae and generally improve field performance of *P. radiata* seedlings. In the first test, *S. luteus* improved field performance but to a lesser degree.

Adverse sites in United States

Beginning in 1973, field tests were conducted in the United States to ascertain the value of mycorrhizae formed by *Pisolithus tinctorius* and other fungi for improving survival and growth of pines on adverse sites. Since most of this data was summarized recently (Marx 1977a), only a few examples will be briefly discussed and updated here. One of our first tests was installed in Kentucky on a very toxic (pH 3.8) coal spoil that had been unsuccessfully planted with pine seedlings several times. Seedlings of *Pinus virginiana* were produced in our nursery with *Pisolithus* and *Thelephora* mycorrhizae using methods described earlier (Marx and Bryan 1975). The seedlings were graded to similar heights and root collar diameters; all had about 75 per cent mycorrhizal development. Seedlings inoculated with *P. tinctorius* had two-thirds of this amount formed by *P. tinctorius*. All other mycorrhizae were formed by *T. terrestris*. The field design was random with five blocks. Test plots within each block were separated by a 4 m non-planted border. After two years only two of the 160 seedlings with *Thelephora* mycorrhiza survived, whereas 78 of the 160 seedlings with *Pisolithus* mycorrhiza survived. More significant was the growth of *Pisolithus* seedlings which produced an average seedling volume* of 130 cm^3 compared to the two *Thelephora* seedlings with an average of 3 cm^3. This volume of

*Seedling volume (cm^3) = (root collar diameter, cm)^2 × height, cm.

Thelephora seedlings was the same as that measured at planting, indicating that these seedlings did not grow during this two-year period.

A similar planting with five blocks was installed on a coal spoil (pH 3.4) in Virginia with *P. taeda* seedlings produced as before. Unfortunately, trees destroyed by vandals precluded accurate assessments of survival. Growth measurements after two years showed that seedlings with *Pisolithus* mycorrhiza has an average seedling volume of 962 cm^3 and those with *Thelephora* mycorrhiza had a volume of 379 cm^3. The last example of a coal spoil study was installed on another toxic (pH 3.9) site in Kentucky. This spoil was unsuccessfully planted twice with nursery seedlings of *P. taeda*. Seedlings of *P. taeda* and *P. echinata* were produced as before with *Pisolithus* and *Thelephora* mycorrhizae and graded to similar sizes and ectomycorrhizal development. The field design was randomized with five blocks. After two years survival was not influenced by treatments, but growth was strongly affected. *Pinus taeda* seedlings with *Pisolithus* mycorrhiza had plot volume indices* of 13 000 cm^3 and those with *Thelephora* had 2000 cm^3. *Pinus echinata* seedlings with *Pisolithus* mycorrhiza had plot volumes of 3600 cm^3 compared to *Thelephora* seedlings with a volume of 700 cm^3 (Marx and Artman 1979).

We prefer this plot volume index (PVI) parameter because it integrates survival, height, and root collar diameters into a single value for comparison. It also represents an accurate measure of the response of all seedlings to treatment. We have found that height measurements alone give poor representations of pine seedling performance.

In all the field tests on coal spoils yearly root evaluations were made. The results confirmed that *P. tinctorius* is ecologically adapted to these harsh sites. Without exception, seedlings with *Pisolithus* mycorrhiza at planting had new roots totally colonized by this fungus. Colonization was so prolific that after the second year hyphal strands were easily detected in the spoil material with the unaided eye as far as 3 m from the young seedlings. Production of sporophores of *P. tinctorius* in the test plots was also prolific. In one *Pisolithus* plot (49 m^2) of *P. taeda*, 83 sporophores were collected during one visit. In contrast, on seedlings that initially had *Thelephora* mycorrhizae new root growth was minimal and only a few were colonized by *Thelephora* at the end of the first year. Many of the original *Thelephora* mycorrhizae (mycorrhizae located on the original root system) were necrotic and only a few visually appeared to be functional. After the second year, a low incidence (three to five per cent) of *Pisolithus* mycorrhizae was detected on newly formed roots on one site; *Thelephora* had spread slowly to new roots on the two other

*Plot volume index, cm^3 (PVI) = (root collar diameter, cm)^2 × height, cm × No. surviving tree seedlings per plot.

sites. Sporophores of *T. terrestris* were only occasionally observed in *Thelephora* plots.

Fourth year data were recently collected and evaluated from the tests on coal spoils in Virginia and the last site in Kentucky. Differences in growth were greater after four years than those measured after two years. One interesting observation was made after the severe winter of 1976–7. Winter scorch of needles was severe on all seedlings on both sites. Seedlings with *Pisolithus* mycorrhiza, however, recovered from this severe needle browning at least six weeks earlier in the spring than seedlings with *Thelephora* mycorrhiza. This earlier recovery undoubtedly gave seedlings with *Pisolithus* mycorrhiza a longer active growth period than seedlings with *Thelephora* mycorrhiza.

A variety of field tests have also been installed on other types of adverse sites (Marx 1977a). Only a few of these will be discussed here. Kaolin spoils are created by strip mining. They are nutrient deficient, reflect sunlight from their light colour, and usually are drought-prone and highly compacted, but most lack the toxic characteristics of coal spoils. In 1975, *P. taeda* seedlings with mycorrhizae formed by either *P. tinctorius, T. terrestris,* or *C. graniforme* were produced in our mycorrhizal fungus-free growth room by Otrosina (1977). Seedlings inoculated with *P. tinctorius* had 85 per cent *Pisolithus* mycorrhizal development. Those inoculated with *Cenococcum* had 20 per cent *Cenococcum* and 30 per cent *Thelephora* (contaminant) mycorrhizae. Seedlings inoculated with *T. terrestris* had 85 per cent *Thelephora* mycorrhizae. These seedlings were outplanted in central Georgia on two different kaolin spoils. Both sites were covered with 7.5 cm of forest soil prior to planting. This is a recommended practice to assist in reclamation of the spoils. Treatments were arranged in a random block design with four blocks. Half of the seedlings were fertilized with 170 g of 10:10:10 NPK fertilizer per seedling at planting and the other half received no fertilizer. Only first year data are available. On one site, seedlings with *Cenococcum* mycorrhizae survived better in the fertilized (83 per cent) than in the non-fertilized (55 per cent) plots, but seedling growth was similar. Survival (65 and 73 per cent) and growth of seedlings with *Thelephora* mycorrhizae were not affected by fertilization. Generally, seedlings with *Cenococcum* grew better than seedlings with *Thelephora* mycorrhiza. In the non-fertilized plots, seedlings with *Pisolithus* mycorrhiza were significantly larger and survived better (73 per cent). Overall seedling growth on the second site was generally greater than growth on the first site. Fertilization significantly improved height growth but specific mycorrhiza had no effect (only *Pisolithus* and *Cenococcum* tested). Root evaluation at the end

of the growing season revealed that all introduced fungi persisted on roots of their respective seedlings. However, on the first site the degree of mycorrhiza developed by each fungus decreased; on the second site, the degree of development was greater for both *Pisolithus* and *Cenococcum* than on the other site. This author also concluded that ectomycorrhizal fungi, such as *P. tinctorius* and *C. graniforme*, ecologically adapted to adverse soil conditions, afford improved survival and growth to pine seedlings on kaolin spoils over seedlings with mycorrhizae formed by fungi such as *T. terrestris*. In earlier work on kaolin spoils (Marx 1977a), hyphal strands of *Pisolithus* were found to spread very rapidly through the spoil material. In one study, spread was so rapid that by the end of the first growing season hyphal strands had grown nearly 2 m from inoculated seedlings and had formed mycorrhizae on control seedlings planted in adjacent rows. The integrity, therefore, of the ectomycorrhizal treatments was lost. This rapid spread between rows brought about changes in subsequent experimental designs. Since 1974, we plant seedlings with different mycorrhizal fungi in discrete plots separated by at least a 2 m non-planted border. Frequently, if space is available, a 4 m strip is left non-planted.

Severely eroded sections of the Copper Basin of Tennessee were also used as outplanting sites. Since the 1840s thousands of hectares of productive forest were decimated by cutting timber and using the wood in heap roasting of mineral ores. This roasting produced SO_2 which further damaged vegetation. Severe sheet erosion followed this destruction of vegetation. Air quality was improved in 1964 and reforestation efforts began, but with little success. The surface soil in most places in the basin is gone, leaving nothing more than exposed parent material. The soil has low levels of available nutrients, high temperatures during summer months, and poor internal water drainage, but does not contain levels of any element toxic to pines. Root examinations of pines planted in the basin reveal two obvious forms of ectomycorrhizae. *Thelephora* mycorrhizae and its sporophores occur sporadically. It was probably introduced on roots of the outplanted seedlings from a nursery. The other type, formed by *Pisolithus*, occurs naturally on pines and oaks on the perimeter of the Basin and was probably introduced by wind-borne basidiospores.

Tests were installed at two locations in the Basin in March 1974 (Berry and Marx 1978). Seedlings of *Pinus taeda* and *P. virginiana* were produced in our microplot nursery with *Pisolithus* and *Thelephora* mycorrhizae as described earlier. All seedlings were graded to similar sizes and degrees of mycorrhizal development. Those inoculated with *Pisolithus* had over two-thirds of the total of 70 per cent mycorrhizal development formed by *Pisolithus* and seedlings inocu-

lated with *Thelephora* had about 70 per cent development by *Thelephora*. Prior to planting, the test sites were levelled and the soil was ruptured (subsoiled) to a depth of 60 cm to destroy hardpans and to allow roots and water to more readily penetrate the soil. Fertilizer (672 kg/ha of 10:10:10 NPK) and dolomitic limestone (4480 kg/ha) were broadcast and disced into the soil of all plots. The trees were planted in a randomized design with three blocks. Each plot was surrounded by a border row of trees and a 2 m non-planted strip. After two years, survival of both pine species (88 to 99 per cent) was not affected by mycorrhizal treatments. *Pisolithus* mycorrhizae significantly increased height and stem diameters of both pine species on one site. Seedling volumes of *P. taeda* were 93 per cent and *P. virginiana* were 90 per cent greater than those of comparable seedlings with *Thelephora* mycorrhiza. Unfortunately excessive experimental variations precluded statistical significance on the second site, even though *Pisolithus* mycorrhiza increased seedling volume by 45 per cent for *P. taeda* and 26 per cent for *P. virginiana* seedlings. Both fungi persisted well on roots, since root evaluations showed that they were still dominant on their respective seedlings. Sporophores of *P. tinctorius* were also produced in great abundance in the *Pisolithus* plots.

The last example of research done on adverse sites is from a borrow pit in the lower piedmont of South Carolina (Ruehle, unpublished data). A borrow pit is an area from which soil was removed (borrowed) for use in construction. This eight hectare site was created in the 1950s by vertical removal of from 1 to 5 m of soil. The resulting surface material had physical and chemical characteristics very similar to those of kaolin spoils. In 1955, the site was planted with nursery seedlings of *P. taeda*. By 1975, most of the trees were less than 2 m tall and very chlorotic; root penetration was very restricted. *Pisolithus* occurred naturally on many of these trees. Prior to study installation in 1975, the trees were removed and the site was levelled. The area was then subsoiled as described earlier. Fertilizers (560 kg/ha of 10:10:10 NPK) and dolomitic limestone (2240 kg/ha) were broadcast on half the plots and the other half received a 1 cm layer of dried sewage sludge. All plots were disced and seeded to grass. In 1976, seedlings of *P. taeda* were grown in styrofoam containers with vermiculite-peat moss substrate. Prior to seeding, the substrate in one-third of the containers was mixed 8:1 with leached, non-dried pure mycelial inoculum of *P. tinctorius*. These seedlings were grown in the greenhouse. One-third were grown in the greenhouse without artificial inoculation for natural colonization by *T. terrestris*. The remaining one-third were grown in the mycorrhizal fungus-free growth room in a non-mycorrhizal condition. All seedlings were

watered as needed and fertilized lightly. After four months, all seedlings, regardless of treatment, were about 10 cm tall. Those inoculated with *Pisolithus* had about 20 per cent mycorrhizae formed by *Pisolithus* and 30 per cent mycorrhizae formed by naturally occurring *T. terrestris*. The second group had a total of 65 per cent mycorrhizal development, all formed by *T. terrestris*. The third group from the growth room lacked mycorrhizae. Seedlings were planted in November 1976 in a randomized design with five blocks. Each plot was surrounded by a border row of seedlings and a 4 m non-planted space.

Only data from one growing season are available. In the sludge amended plots, survival was 90 per cent for pine seedlings with a mixture of *Pisolithus* and *Thelephora* mycorrhiza, 75 per cent for those with just *Thelephora* mycorrhiza, and only 62 per cent for non-mycorrhizal seedlings. The PVIs were 1702 cm^3 for seedlings with *Pisolithus* mycorrhiza, 361 cm^3 for those with *Thelephora*, and only 104 cm^3 for those without mycorrhiza. The differences due to *Pisolithus* mycorrhiza in comparison to *Thelephora* and non-mycorrhizal seedlings represent increases of 372 and 153 per cent, respectively. In the fertilized plots similar results were obtained but they were not as striking. Survival was 98, 89, and 88 per cent, respectively, for seedlings with *Pisolithus*, *Thelephora*, or no mycorrhizae. The corresponding PVIs were 75, 61, and 27 cm^3. Root evaluation data are not available at this time.

These results not only show that seedlings with mycorrhizae survive and grow better than seedlings lacking mycorrhizae, but they also show that seedlings with *Pisolithus* mycorrhiza are better adapted to adverse soils in borrow pits even after amendments with sludge or fertilizers.

We can conclude from these field studies on adverse sites that reclamation and reforestation of such sites can be expedited by using pine seedlings tailored with mycorrhizae formed by fungi capable of growing under adverse conditions. Thus, the planting of seedlings with root systems physiologically and ecologically adapted to accommodate the adversities of the planting site can be an important biological tool in reforestation. In reality, however, sites such as coal and kaolin spoils and borrow pits are not the only adverse sites created by the activities of man. Many reforestation sites, especially those which have been intensively site prepared (stump shearing, root raking, slash removal, burning, discing, etc.), are temporarily adverse (Schultz 1977). Until vegetation is re-established by either natural or artificial means, the mineral soils are often exposed and subject to broad fluctuations of temperature, moisture, and fertility, as well as to erosion and compaction (Haines, Maki, and Sanderford 1975).

These are adverse soil conditions, however temporary, to which root systems of newly planted seedlings will be exposed. If these soil conditions are extreme, the survival and early growth of seedlings with mycorrhizae formed by nursery adapted fungi, i.e. *Thelephora*, may be unduly affected. This point could explain certain reforestation failures in the past on sites which have been intensively prepared.

Routine sites in United States

After considering these points, we installed several studies on routine reforestation sites to compare the effects of *Pisolithus* and *Thelephora* mycorrhiza on establishment and early growth of various pines. Since 1974, nearly 75 000 seedlings have been experimentally outplanted on a variety of reforestation sites. In most studies, seedlings were graded so that different amounts of *Pisolithus* and *Thelephora* mycorrhizae were treatment variables. The following are results of one such study (Marx, Bryan, and Cordell 1977). Pine seedlings were produced in Florida and North Carolina nurseries with *Pisolithus* and naturally occurring *Thelephora* mycorrhizae. The nursery phase of this study was described earlier (Marx *et al.* 1976). The use of basidiospores and pure mycelial inoculum of *P. tinctorius* resulted in seedlings with different quantities of *Pisolithus* mycorrhizae. In the Florida nursery, seedlings of *P. taeda, P. elliottii* var. *elliottii,* and *P. clausa* were graded to equal size and to 65 per cent mycorrhizal development. Those inoculated with mycelial inoculum had three-quarters of this 65 per cent formed by *Pisolithus* and those inoculated with basidiospores had about one-third of this amount formed by *Pisolithus.* Remaining mycorrhizae on these seedlings and the control seedlings were formed mainly by *Thelephora terrestris*. Three sites in Florida, recently clearcut of pines, were site prepared and all slash was burned exposing mineral soil. One site was a deep sand ridge; one was a palmetto flatwood. The third site was also a flatwood site but it was planted to only *P. elliottii* var. *elliottii* with abundant *Pisolithus* or *Thelephora* mycorrhizae. No intermediate amount of *Pisolithus* was used. This study also involved a fertility variable of 90 kg/ha of both nitrogen and phosphorus.

In the North Carolina nursery, seedlings of *P. taeda, P. virginiana,* and *P. strobus* were graded to equal size and also to 75 per cent mycorrhizal development. Generally, seedlings inoculated with mycelial inoculum had at least eight-tenths of the 75 per cent total development formed by *Pisolithus* and those inoculated with basidiospores had between one-tenth to two-thirds of the total mycorrhizae formed by *Pisolithus.* The two test sites in North Carolina were cleared of pine and oak, site prepared, and all slash was piled and burned. One site was considered a good reforestation site because

of the presence of 25 cm of top soil. The other was considered poor because it was an eroded slope.

Seedlings on four sites were planted in a random design with five blocks. The fertilizer–mycorrhiza study had only three blocks. Plots within blocks were separated by at least a 3 m non-planted strip.

After two years, seedlings with the greatest quantity of *Pisolithus* mycorrhizae at planting generally survived and grew better than seedlings with the same amount of mycorrhizae formed by *T. terrestris*. Seedlings with less *Pisolithus* mycorrhizae were usually intermediate between these two seedling groups in survival and growth. In North Carolina, abundant *Pisolithus* mycorrhizae significantly increased PVI of both *P. virginiana* and *P. taeda* by about 25 per cent on the good site and by about 50 per cent on the poor site in comparison to seedlings with only *Thelephora* mycorrhizae. *Pinus taeda* seedlings with less *Pisolithus* and more *Thelephora* mycorrhizae (from basidiospores) on the better site had a 30 per cent greater PVI than did the *Thelephora* seedlings; they were not different on the poor site. Greater differences occurred on seedlings of *P. strobus*. Those with abundant *Pisolithus* mycorrhizae had five times greater PVI than seedlings with *Thelephora*. This test with *P. strobus* is somewhat unusual since these seedlings were outplanted after only one growing season in the nursery instead of the normal two growing seasons.

In Florida, the results were similar. On both sites *P. taeda* and *P. elliottii* var. *elliottii* seedlings with abundant *Pisolithus* mycorrhizae had significantly greater PVI (16 to 51 per cent) than *Thelephora* seedlings. On the palmetto flatwood, *P. taeda* seedlings with only one-third of the complement of mycorrhizae formed by *Pisolithus* also had a PVI that was 46 per cent greater than *Thelephora* seedlings. The intermediate amount of *Pisolithus* on seedlings of *P. elliottii* var. *elliottii* did not have an effect on growth. The most significant results were obtained with *P. clausa*. Seedlings with abundant *Pisolithus* had PVIs on both sites that were from 270 to 445 per cent greater than seedlings with *Thelephora*. The greatest difference in survival and growth occurred on the flatwood site which is considered off-site for *P. clausa*. In the fertility study, *P. elliottii* var. *elliottii* seedlings with *Pisolithus* mycorrhiza in the fertilized plots were the same size as seedlings with *Thelephora*. However in the non-fertilized plots, seedlings with *Pisolithus* mycorrhiza had a PVI that was 175 per cent greater than comparable seedlings with *Thelephora* mycorrhiza. Yearly root evaluation for two years of representative seedlings on the various sites revealed that *Pisolithus* persisted well, especially on those sites where it stimulated the most seedling growth. On all sites, particularly the better ones, other ectomycorrhizal fungi such as *Cenococcum graniforme* or an unidentified *Rhizopogon* were

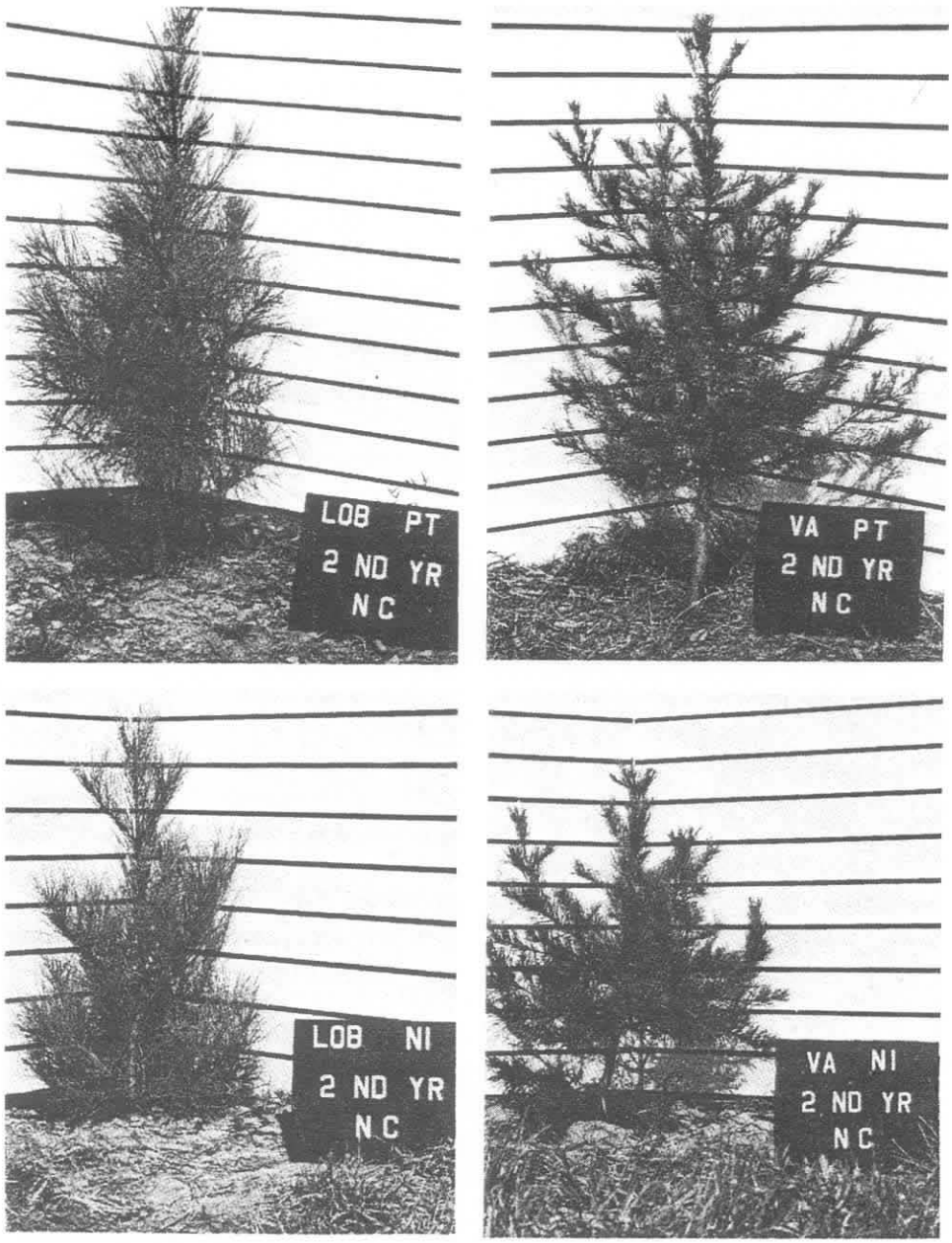

Fig. 2.1. Growth response of pines to specific ectomycorrhizae. Upper left and right photographs are loblolly and Virginia pine seedlings after two years on a routine reforestation site in North Carolina, USA, that had abundant *Pisolithus tinctorius* mycorrhizae on roots at planting time. Lower left and right photographs are corresponding control seedlings that had abundant naturally occurring *Thelephora terrestris* mycorrhizae on roots at planting time. Horizontal lines in background are spaced 10 cm.

observed on new roots of various seedlings regardless of initial mycorrhizal conditions. Sporophores of *P. tinctorius* were also observed in many *Pisolithus* plots. We obtained fourth year data from these seedlings recently (Marx, unpublished data). The differences in growth rate are either greater after the fourth growing season or are approximately the same as those at the end of the second season.

Krugner (1976) grew seedlings of *P. taeda* in a nursery soil of different fertility inoculated with *P. tinctorius, T. terrestris*, or a mixture of the two fungi. As discussed earlier, *Pisolithus* in this study formed more mycorrhizae in fertilized than in non-fertilized soil. Selected seedlings were outplanted on two recently prepared sites in the coastal plain of North Carolina. Only data from the first growing season is available. On one site, excessive weed competition and high populations of indigenous ectomycorrhizal fungi apparently eliminated the effects of the inoculation and fertility treatments from the nursery. The other site was considered poor and seedlings with the greatest amount of *Pisolithus* mycorrhizae had up to 19 per cent better survival, 63 per cent more height growth, and 15 per cent larger stem diameters than seedlings with only *Thelephora* mycorrhiza.

These results indicate that under the temporarily adverse situations caused by tree removal and site preparation of routine reforestation sites, pine seedlings may survive and grow faster if they have abundant mycorrhizae formed by a fungus, such as *P. tinctorius*, which is ecologically adapted to adverse conditions. Apparently, fertilization reduces the adverse situation to such a degree that seedlings with *Thelephora* mycorrhizae can survive and grow as well as those with *Pisolithus*. Our results also show that the more *Pisolithus* mycorrhizae seedlings have on their roots at planting, the more benefit they derive from this specific mycorrhizal association, especially on the poorer reforestation sites. Perhaps the ecological adaptation to poor soil conditions allows *P. tinctorius* a competitive advantage over other ectomycorrhizal fungi for colonization of new feeder roots. On better reforestation sites the other fungi may be more competitive and aggressive than *Pisolithus*. We have found from other field experiments that seedlings with *Thelephora* mycorrhizae in non-stress situations survive as well and grow better than seedlings with *Pisolithus* mycorrhiza. These observations coincide very well with what we think we know about the biological significance of certain ecological adaptability traits of these fungi.

Results from these various field studies show that specific ectomycorrhizal fungi can improve initial field performance of tree seedlings on good and poor sites. Some fungi appear to increase tolerance of the seedlings to extremes in soil environment, whereas others appear to enhance absorption of certain nutrients, such as

phosphorus, from the soil. In all probability, many of these fungi share certain traits which act in concert to increase survival or early growth of tree seedlings. Unfortunately, there is no data to show whether these early growth effects have any influence on the final volume of wood harvested at the end of the normal rotation. Only long-range studies can furnish information of this type.

Conclusion

There is no doubt that a variety of proven methods are available to ensure the development of ectomycorrhizae on forest tree seedlings for the establishment of man-made forests. Certain methods have more advantages than others. Some methods, such as the use of pure mycelial inoculum, have more biological advantages than others, but a great deal more research must be done. There is sufficient information, however, to conclude that pure cultures of certain fungi, such as *Suillus granulatus, Rhizopogon luteolus*, and *Pisolithus tinctorius* can be used to assure good survival and growth of tree seedlings on a variety of sites. These represent only the beginning of the practical concept, however. When one considers the millions of hectares of potential exotic forests which should be established in Third World nations, as well as the millions of hectares of former forest lands awaiting artificial regeneration throughout the world, the importance of the selection, propagation, manipulation, and management of superior strains or species of mycorrhizal fungi as a forest management tool is paramount. Research so far has only revealed the tip of the iceberg in regard to potential use in world forestry. There still remains a tremendous reservoir of basic and practical information which must be revealed if these fungi are to be managed and, therefore, fully utilized in forest regeneration.

The introduction of ectomycorrhizal fungi into various parts of the world to establish exotic forests has expanded the geographic range of these fungi throughout the world (Mikola 1969, 1970, 1973) Although many species probably died, numerous fungi are currently thriving in areas far distant from their original habitat. These fungi, either individually or as a group, have a tremendous capacity to adapt to different environments. Once techniques have been perfected for use of pure cultures and adequate quantities of inoculum are available, the specific fungi should be tested on tree seedlings over a wide range of environmental conditions encountered in forestation throughout the world. There is not sufficient information available in the world literature on the use of a specific fungus to even remotely suggest where it can or cannot benefit a specific tree species in a given locality. Even though the effect of a given

fungus may only be temporary, its short term influence may make the difference between initial success or failure of seedling establishment. There are several botanical precedents for this idea of testing an organism over a spectrum of environmental conditions beyond those present in its natural habitat. One such example is *Pinus radiata*. Its natural range is restricted to about 4500 hectares along the coast of California. Since the mid-1800s this tree has been planted successfully throughout the world, including such countries as Australia, New Zealand, Chile, Bolivia, Spain, Ireland, and several African nations. By 1958, over 623 000 hectares had been planted (Scott 1960). In many of these countries it has become the major commercial forest tree. There is little doubt that these forests are established today because foresters took a broad ecological view of the potential range of *Pinus radiata*. Researchers on mycorrhizae should also approach the use of specific ectomycorrhizal fungi in world forestry from a broad ecological view until results from research dictate otherwise. Let us determine the biological and practical significance of a given ectomycorrhizal fungus to forest productivity not by supposition, but by facts obtained by using scientific rules of proof.

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